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Identification of future target therapy in peritoneal carcinomatosis: the role of fibrin and heparanase

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the Paris Diderot University."

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"Being a scientist means living on the borderline between your competence and your incompetence. If you always feel competent, you aren't doing your job."

Carlos Bustamante

To Say Cancer is a Challenge but Not an End

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SUMMARY

ABSTRACT

Several gastrointestinal and gynecological tumors have the potential to spread and develop in the peritoneal cavity. Carcinomatosis is characterized by dissemination of tumor cells within the peritoneum. The peritoneum is covered by a layer of mesothelial cells forming a non-adhesive protective surface involved in fluid transport as well as in antigen presentation, coagulation and fibrinolysis. In pathological conditions, the peritoneal wall loses its biological properties and becomes a favorable ground for the implantation of tumor cells.

The influence of chemotherapy on phenotypic changes in tumor cells of cancerous nodules was studied. Before treatment, all neuronal markers except NSE and S100 protein were found negative in primary tumors. In the proliferative zone of post-operative samples, NSE and S100 protein markers persist with any other neuronal markers. In contrast, in degenerative non-proliferative zone, we found all neuronal markers except synaptophysine and dramatically decreased infiltrated immune cells. These results are in favors of differentiation of poorly differentiated ovarian cancer cells to other tissue with no proliferation potential.

We reported for the first time that the pattern of expression of thrombopoietin (TPO) gene in ovarian cancer cells (OVCAR-3) is similar to that observed in the liver. The supernatant of OVCAR-3 cells can replace exogenous TPO and inhibit apoptosis of the TPO-dependent cell line (Ba / F3) suggesting that TPO produced is functional.

For the first time, we studied the role of heparanase secreted by cancer cells in epithelial to mesenchymal transition (EMT) and as an inducer of fibrosis. High expression of heparanase (HPSE) mRNA and protein was found in gastric signet ring cell adenocarcinoma (SRCA) tumor and ascites as well as in KATO-III cell lines. Beside of collagen-I, growth factors (TGF-β1 and VEGF-A, except FGF-2) and EMT markers (Snail, Slug, Vimentin, α-SMA and Fibronectin, except E-cadherin) were found higher in main nodule of SRCA as compared to peritumoral sites. Among MDR proteins involved in chemoresistance, MDR-1 and LRP (lung resistance protein) were strongly expressed in tumor cells. After treatment of KATO-III with a heparanase inhibitor (suramin), cell proliferation and markers associated with EMT, in addition to the expression of collagen-1, were decreased. HPSE as well as stem cell

markers decreased when cells were incubated with agents inducing cell differentiation.

We explored the effect of microenvironment on mesothelial layer hemostasis and fibrin deposition involved in adhesion and dissemination of cancer cells. Our results demonstrated that the microenvironment in carcinomatosis leads to changes in the mesothelial cells: 1) modification of their morphology, 2) increase of EMT markers, 3) modification of their behavior related to increased secretion of neprilysin, MMP2, TF as well as different cytokines, 4) detachment of peritoneal cells, and 5) increase of interleukins 6 and 8, HGF (hepatocyte growth factor) expression of mRNA and protein responsible for leukocyte chemotaxis. Cancer cells clusters in ascites have been shown to be associated with fibrin deposition. We also observed that expansion of fibrin fiber filaments inhibits the adhesion of cancer cells to the peritoneal surface, leading to the appearance of cellular clusters.

RESUME

Plusieurs tumeurs gastro-intestinales et gynécologiques ont le potentiel de se disséminer et de se développer dans la cavité péritonéale. La carcinose est caractérisée une dissémination des cellules tumorales à l'intérieur du péritoine. Le péritoine est recouvert par une couche de cellules mésothéliales formant une surface protectrice non-adhésive impliquée dans le transport des fluides ainsi que dans la présentation de l'antigène, la coagulation et la fibrinolyse. Dans les conditions pathologiques, la paroi péritonéale perd ses propriétés biologiques et devient un terrain favorable pour l'implantation des cellules tumorales.

L'influence de la chimiothérapie sur les modifications phénotypiques dans les cellules tumorales des nodules cancéreuses a été étudiée. Avant le traitement, tous les marqueurs neuronaux à l'exception des protéines NSE et S100 ont été trouvés négatifs dans les tumeurs primaires. Dans les échantillons post-opératoires et après chimiothérapie, au niveau de la zone prolifération, les marqueurs protéiques NSE et S100 persistent. En revanche, dans la zone non prolifération dégénérative, nous avons trouvé tous les marqueurs neuronaux sauf synaptophysine et diminué considérablement les cellules immunitaires infiltrées. Ces résultats sont en faveur de la différenciation des cellules cancéreuses de l'ovaire peu différenciées en d'autres tissus sans potentiel de prolifération.

Nous avons rapporté pour la première fois que le profil d'expression du gène de la thrombopoïétine (TPO) dans les cellules cancéreuses de l'ovaire (OVCAR-3) est similaire à celui observé dans le foie. Le surnageant des cellules OVCAR-3 peut remplacer la TPO exogène et inhiber l'apoptose de la lignée cellulaire TPO-dépendante (Ba / F3) suggérant que la TPO produite est fonctionnelle.

Pour la première fois, nous avons étudié le rôle de l'héparanase sécrétée par les cellules cancéreuses dans la transition épithéliale à mésenchymateuse (EMT) et comme un inducteur de la fibrose. Une expression élevée de l'ARNm de l'héparanase (HPSE) et de la protéine a été trouvée dans l'adénocarcinome gastrique signet ring cell (SRCA) et dans les ascites ainsi que dans les lignées cellulaires KATO-III. À côté du collagène I, des facteurs de croissance (TGF-β1 et VEGF-A) et des marqueurs de transition mésenchymateuse épithéliale (EMT) (vimentine, α-SMA et fibronectine, sauf E-cadhérine) ont été trouvés plus élevé dans le nodule principal de SRCA que

dans les sites péritumoraux. Parmi les protéines MDR impliquées dans la résistance à la chimiothérapie, MDR-1 et LRP (protéine de résistance pulmonaire) étaient fortement exprimées dans les cellules tumorales. Après le traitement de KATO-III avec un inhibiteur de l'héparanase (suramine), la prolifération cellulaire et les marqueurs associés à l'EMT, outre l'expression du collagène-1, ont été diminués. HPSE ainsi que les marqueurs de cellules souches ont diminué lorsque les cellules étaient incubées avec des agents induisant la différenciation cellulaire.

Nous avons exploré l'effet du microenvironnement sur l'hémostase de la couche mésothéliale et le dépôt de fibrine impliqués dans l'adhésion et la dissémination des cellules cancéreuses. Les résultats démontrent que le microenvironnement dans la carcinomatose entraîne des modifications des cellules mésothéliales: 1) modification de leur morphologie, 2) augmentation des marqueurs EMT, 3) modification de leur comportement lié à une augmentation de sécrétion de neprilysin, MMP2, TF ainsi que différentes cytokines, 4) détachement des cellules du péritoine, et 5) augmentation des interleukines 6 et 8, de l'HGF (facteur de croissance des hépatocytes) et augmentation de expression de l'ARNm de la protéine responsable du chimiotactisme de leucocytes. Des amas de cellules cancéreuses présents dans l'ascite se sont révélés associés à des dépôts de fibrine. Nous avons également observé que l'expansion des filaments de fibre de fibrine inhibe l'adhérence des cellules cancéreuses à la surface péritonéale, entraînant l'apparition d'amas cellulaires dans l'ascite.

ABBREVIATIONS

ABC	ATP-binding cassette
aPC	Activated protein C
APL	Acute promyelocytic leukemia
α-SMA	α-Smooth muscle actin
ATRA	All-trans retinoic acid
BM	Basement membrane
BMP	Bone morphogenetic protein
CAFs	Carcinoma Associated Fibroblasts
CALLA	Common Acute Lymphoblastic Leukemia
Col-I	Collagen Type I
Col-IV	Collagen Type IV
CAPD	Continuous ambulatory peritoneal dialysis
CSCs	Cancer stem cells
CTL	Cytotoxic T-lymphocyte
delta Ct	Threshold Cycle
EC	Endothelial cells
E-Cad	E-cadherin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	Epithelial Membrane Antigen
EMT	Epithelial mesenchymal transition
EPCs	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
EPCRs	Soluble EPCR

FGF-2	Fibroblast growth factor 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gastric Cancer
HBD-1	Heparanase binding domain-1
HGF	Hepatocyte growth factor
HPSE	Heparanase
HUVEC	Human umbilical vein endothelial cell
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IKKα	IκB Kinase α
IL	Interleukin
JAK	Janus kinase
kDa	Kilo daltons
LECs	Lymphatic endothelial cells
LIC	Leukocyte-initiating cells
TNF-α	Tumor necrosis factor-Alpha
LRP	Lung resistance protein
MAPK	Mitogen-activated protein kinases
MDR	Multidrug resistance protein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRP-1	Multidrug resistance protein-1
MSCs	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NPC	Nasopharyngeal carcinoma

NSAIDs	Non-steroidal Anti-Inflammatory Drugs
PB	Base pair
PC	Peritoneal carcinomatosis
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
s.c	Subcutaneously
SCS-3	Suppressor of cytokine signaling-3
siRNA	Small (or short) interfering RNA
SLNs	Sentinel lymph nodes
SP	Side population
SRCA	Signet ring cell adenocarcinoma
STAT	Signal transducer and activator of transcription
TAMs	Tumor-associated macrophages
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor β
TME	Tumor microenvironment
TPO	Thrombopoietin
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase receptor
VEGFA	Vascular Endothelial Growth Factor A
WHO	World Health Organization

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INTRODUCTION

Chapter 1: Cancer Statistics

1. Cancer

Cancer is not a new disease and has afflicted people throughout the world. The word cancer came from a Greek words karkinos to describe carcinoma tumors by a physician Hippocrates (460–370 B.C), but he was not the first to discover this disease. Some of the earliest evidence of human bone cancer was found in mummies in ancient Egypt and in ancient manuscripts dates about 1600 B.C. The world's oldest documented case of cancer hails from ancient Egypt in 1500 BC and it was recorded that there was no treatment for the cancer, only palliative treatment. According to inscriptions, surface tumors were surgically removed in a similar manner as they are removed today [1].

Cancer is the second leading cause of death in the world after cardiovascular diseases in both more and less economically developed countries; the burden is expected to grow worldwide due to the growth and aging of the population, particularly in less developed countries, in which about 82% of the world's population resides [2]. There are limited published data on recent cancer incidence and mortality trends worldwide.

Carcinogenesis is a multistep process that results from genetic alterations that underlie the transformation of normal cells into malignant derivatives. In fact, the genome of tumor cells is altered at several sites as a result of point mutations or changes in chromosome integrity. It is now accepted that cancer arises from a succession of genetic alterations that confer growth advantages leading to the progressive conversion of normal cells into cancer cells [3].

2. Gastric cancer

Stomach cancer is the fifth most common cancer worldwide, with an estimated 952 000 new cases (7% of total cancer incidence) and 723 000 deaths (9% of total cancer mortality) in 2012. Almost three quarters of the new cases occurred in Asia, and more than two fifths occurred in China. There is a 10-fold international variation in stomach cancer incidence; rates in men are approximately double those observed in women. The highest age-standardized incidence rates are in East Asia and central and Eastern Europe. Incidence rates tend to be relatively low in Africa and in North America. The case fatality rate is lower in countries with high levels of human development (overall mortality-to-incidence ratio, 0.65) than in countries at low or medium levels of human development (0.83). Over the past 50 years, incidence and mortality rates of the non-cardia type of gastric cancer have been uniformly decreasing in almost all countries; rates of gastric cardia cancer have, however, been stable or increasing in the past two to three decades [4].

There is several classification systems used for stomach cancer around the world. 2 main classification systems are used.

2.1. Lauren classification

The Lauren classification is based on how the gastric tissue looks and behaves when examined under a microscope. This is the system most often used to describe how adenocarcinoma tumors, the most common type of stomach cancer, look and behave.

The Lauren classification divides adenocarcinoma of the stomach into 2 main types[5]:

- intestinal type – Tumor cells are well differentiated, grow slowly and tend to form glands. This type is found more often in men than in women and occurs more often in older people.
- diffuse type – Tumor cells are poorly differentiated, behave aggressively and tend to scatter throughout the stomach (rather than form glands). This type spreads to other parts of the body (metastasizes) much quicker than intestinal type tumors. The diffuse type occurs equally among men and women and tends to develop at a younger age than the intestinal type.

In this system there may also be a mixed type of adenocarcinoma of the stomach, made up of both intestinal and diffuse types.

2.2. WHO classification

The 2010 World Health Organization (WHO) classification goes into more detailed groups than the Lauren classification[6]. There are 5 main types of stomach cancer in the WHO classification.

- tubular adenocarcinoma – This is made up of different sized small branching tubes (tubules).
- papillary adenocarcinoma – This tumour grows outward from the stomach wall and contains finger-like growths that stick out into the stomach cavity. The cells tend to look and behave much like normal cells.
- mucinous adenocarcinoma – There is a lot of mucin (the main substance in mucus) outside of the cancer cells.
- poorly cohesive carcinomas (including **signet ring cell carcinoma** and others) – These are arranged into clumps of cancerous cells.
- mixed carcinoma – This contains a mix of types of adenocarcinomas of the stomach.

2.3. Gastric Cancer in Europe

Gastric cancer is the sixth most common cancer in Europe, with more than 139,000 new cases diagnosed in 2012 (4% of the total). In Europe (2012), the highest World age-standardized; the incidence rates for stomach cancer are in Belarus for men and Albania for women; the lowest rates are in Sweden for both men and women. UK stomach cancer incidence rates are estimated to be the fifth lowest in males in Europe, and fourth lowest in females [7]. These data are broadly in line with Europe-specific data available elsewhere [8].

2.4. Gastric signet ring cell adenocarcinoma (SRCA)

Signet-ring cell carcinoma (SRCA), a unique subtype of mucin-producing adenocarcinoma, can arise from virtually all organs. However, more than 90% of cases of human SRCAs arise from the colon, stomach and breast [9-11]. It has been reported that 29% of patients with gastric cancer had a signet ring cell type tumor

histology [9]. Gastric signet ring cell carcinoma (SRCA) is defined as an adenocarcinoma in which the majority (>50%) consists of isolated or small groups of malignant non-cohesive cells containing intracytoplasmic mucin [12]. In SRCA, stomach wall becomes thickened, rubbery and loses its ability to distend. The stomach assumes a "leather bottle" shape. It is most often seen in adenocarcinoma of the stomach. The term is often used synonymously with diffuse adenocarcinoma of the stomach. Gastric SRCA progression preferentially involves lymph nodes and peritoneal surfaces [13]. Signet ring cell cancer of the stomach occurs at a relatively high frequency in females and in young patients [9].

A down-regulation of epithelial cadherin is essential for the initiation, and progression of gastric signet ring cell cancer cells. Once gastric cells lose E-cadherin, they have an increase in motility due to epithelial-mesenchymal transition. A strong correlation in the mutation of Snail, Slug, and Twist as well as an activation of the phosphatidylinositol 3 kinase (PI3K)/AKT axis, Wnt/β-catenin signaling pathway, and transforming growth factor β have been found to be associated with the pathogenesis of gastric signet ring cell cancer [14].

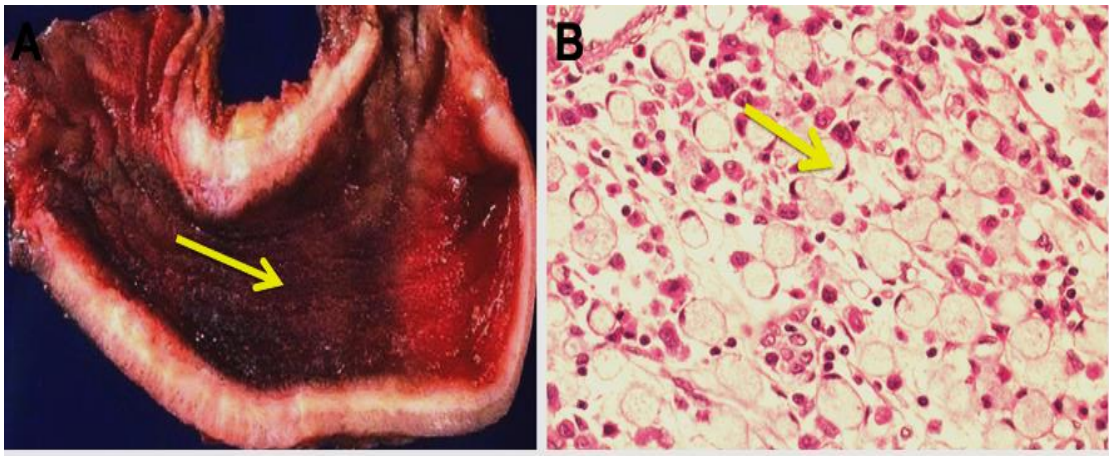

Figure 1: Histopathology of gastric signet ring cell adenocarcinoma.
A stomach with thickened and rubbery wall having a "leather bottle" shape as shown at the arrow (A), Signet ring cell pattern of adenocarcinoma in which the cells are filled with mucin vacuoles that push the nucleus to one side, as shown at the arrow (B).

2.5. Fibrosis induced by SRCA

Gastric signet ring cell adenocarcinoma is characterized by carcinoma cell proliferation and infiltration with extensive fibrosis in the stroma," metastasizes to the peritoneum frequently. Fibrosis is a complex tissue disease whose predominant characteristics are the excessive and abnormal deposition of extracellular matrix (ECM) components [15, 16], that may affect various organs, including lung, stomach, liver, kidney and skin. TAKENOSHITA, S., et al., investigated histochemical relation between the mucin production and the fibrous reaction in SRCA. They found alterations in mucin production during the genesis of signet ring cells [17]. It has been reported that TGF-β1 stimulates the fibrotic changes in SRCA [18]. It has been reported that TGF-β1 molecule secreted in culture media of KATO-III cell line stimulated collagen synthesis for interstitial fibrosis which was later confirmed by immunohistochemically [18].

3. Ovarian Cancer

Ovarian cancer begins in the ovaries. Ovaries are reproductive glands found only in females (women). The ovaries produce eggs (ova) for reproduction. The eggs travel through the fallopian tubes into the uterus where the fertilized egg implants and develops into a fetus. The ovaries are also the main source of the female hormones estrogen and progesterone. One ovary is on each side of the uterus in the pelvis.

The ovaries are made up of 3 main kinds of cells. Each type of cell can develop into a different type of tumor:

- Epithelial tumors start from the cells that cover the outer surface of the ovary. Most ovarian tumors are epithelial cell tumors.
- Germ cell tumors start from the cells that produce the eggs (ova).
- Stromal tumors start from structural tissue cells that hold the ovary together and produce the female hormones estrogen and progesterone.

3.1. Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is a common gynecologic cancer in developed countries, and is either the most common or second most common gynecologic

malignancy [19]. Neoplasms from the surface epithelium of the ovary exhibit a variety of Müllerian-type cells, including serous, mucinous, endometrioid, and clear cell, reflecting a common pathway in embryological development.

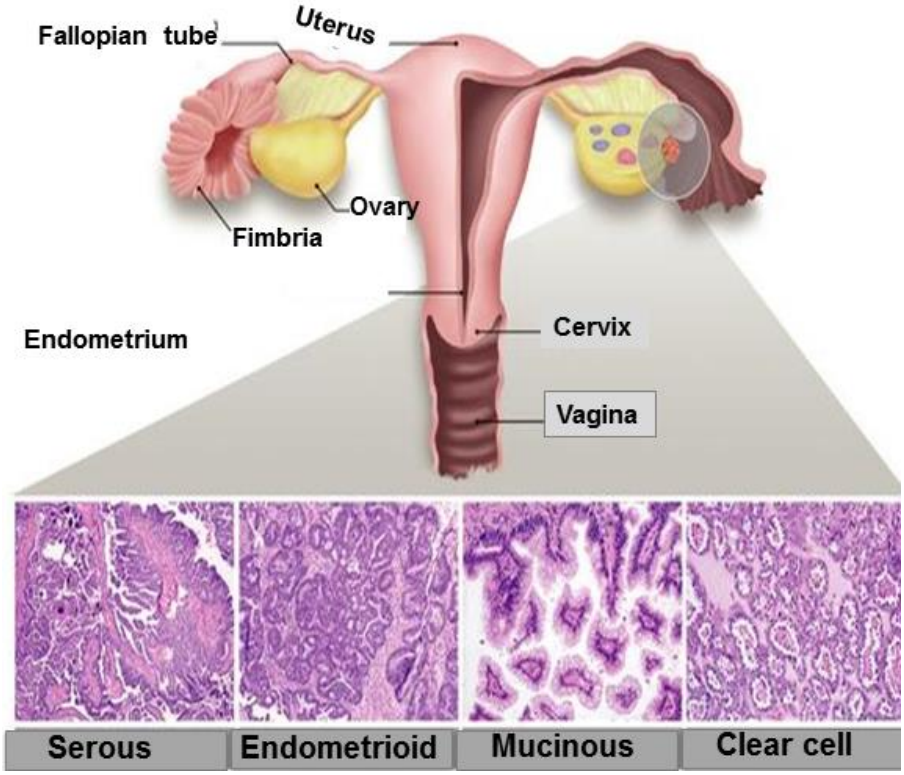

Figure 2: Histological subtypes of epithelial ovarian cancer.

In the western world, EOC is the most lethal gynecologic cancer, accounting for more deaths than endometrial and cervical cancer combined. Spread of the disease via the lymphatics and by peritoneal implantation is not associated with any specific signs or symptoms, and the vast majority of women are diagnosed with disseminated intraperitoneal carcinomatosis (FIGO Stage III). Also contributing to the high mortality is the advanced age at diagnosis (median 63 years), with an increase after menopause. While ultrasound and computerized tomograms are useful in definition of sites of bulk disease, surgical evaluation is necessary for accurate staging and to remove large metastases (cytoreduction). Due to the propensity for diffuse small-volume disease, surgery is rarely able to render patients disease free, and postoperative chemotherapy is usually required. While overall mortality rates have remained relatively constant for the past two to three decades, five-year survival rates have increased from 30% in the 1960s to over 50% in the past several years [20].

3.1.1. Etiology and cellular mechanism of epithelial ovarian cancer

Consistent epidemiological data indicate that the risk of EOC increases with the number of ovulatory events. For the last few decades, two major theories, the incessant ovulation [21] and the gonadotropin hypotheses [22], have been proposed to explain the same epidemiological data [23]. The incessant ovulation hypothesis postulates that the repetitive wounding and recurring cell proliferation in postovulatory repair of the ovarian surface epithelium result in mutations accumulating in the epithelial cells and ultimately tumor formation. This straightforward and conceptually obvious explanation easily gained acceptance. Support for this concept was also provided by laboratory experiments demonstrating spontaneous transformation of rat ovarian surface epithelial cells following pronlonged subculturing [24]. The passaging of the cells in culture mimics repeated injury and proliferative repair of ovarian surface epithelium due to incessant ovulation. Recent experimental evidence supports the idea that higher ovulatory activity is associated with more inclusion cysts and other changes in the ovarian surface, such as invaginations. It has been suggested that these inclusion cysts are a fertile environment for ovarian cancer development [25]. In support of this hypothesis, many (but not all) studies of the ovaries of ovarian cancer-prone individuals, i.e., women with a family history of ovarian cancer and/or a deleterious BRCA1 or BRCA2 mutation, have reported more changes in their surface epithelium than control ovaries [26].

The gonadotropin theory postulates that the surges of pituitary gonadotropins that initiate each ovulation and persist in high levels for years following menopause also stimulate the ovarian surface epithelial cells and induce cell transformation. Abundant epidemiological data and animal models exist to support this idea [22]. Gonadotropins have unremarkable effects on ovarian surface epithelial cells in culture. Thus, inflammation of the ovarian epithelium was suggested as a mechanism by which gonadotropin stimulation and ovulation contribute to ovarian cancer risk [27], since inflammation is a well-known factor contributing to cancer [28]. Ovulation is an inflammatory- like process involving multiple cytokines and proteolytic enzymes, and their actions ultimately lead to tissue rupture [29]. Inflammation can also provide an explanation for the increased risk associated with talc and asbestos exposure,

endometriosis and pelvic inflammatory disease, and mumps viral infection. The ovarian epithelial inflammation caused by ovulation or other factors may contribute to cancer risk by increasing mutations in epithelial cells, as suggested [30].

4. Global cancer statistics

Cancer constitutes an enormous burden on society in more and less economically developed countries alike. The occurrence of cancer is increasing because of the growth and aging of the population, as well as an increasing prevalence of established risk factors such as smoking, overweight, physical inactivity, and changing reproductive patterns associated with urbanization and economic development. Based on GLOBOCAN estimates, about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide [31]. Over the years, the burden has shifted to less developed countries, which currently account for about 57% of cases and 65% of cancer deaths worldwide. Lung cancer is the leading cause of cancer death among males in both more and less developed countries, and has surpassed breast cancer as the leading cause of cancer death among females in more developed countries; breast cancer remains the leading cause of cancer death among females in less developed countries. Other leading causes of cancer death in more developed countries include colorectal cancer among males and females and prostate cancer among males. In less developed countries, liver and stomach cancer among males and cervical cancer among females are also leading causes of cancer death. Although incidence rates for all cancers combined are nearly twice as high in more developed than in less developed countries in both males and females, mortality rates are only 8% to 15% higher in more developed countries. This disparity reflects regional differences in the mix of cancers, which is affected by risk factors and detection practices, and/or the availability of treatment. Risk factors associated with the leading causes of cancer death include tobacco use (lung, colorectal, stomach, and liver cancer), overweight/obesity and physical inactivity (breast and colorectal cancer), and infection (liver, stomach, and cervical cancer) [2].

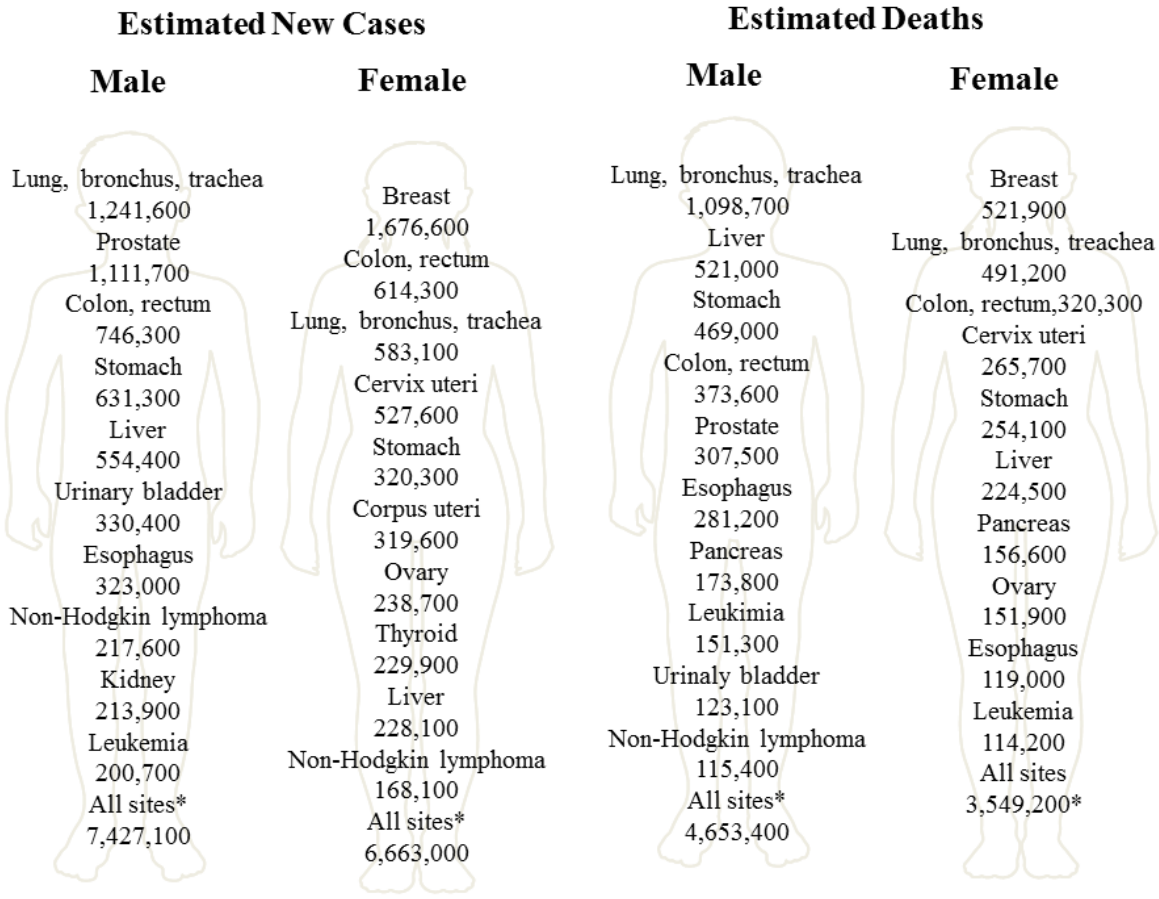

Figure 3: Estimated New Cancer Cases and Deaths Worldwide by Sex and Level of Economic Development [2]

*Excluding non-melanoma skin cancers. Source: GLOBOCAN 2012

5. European Union statistics related to cancer

Europe carries a significant load of the global burden, with one quarter of the global burden of cancer observed in Europe in 2008 despite a total population that comprises one-ninth of the world's population [32, 33].

The timely and comparative situation analysis of the cancer burden in Europe reveals variations in incidence and mortality rates. These reflect variability in the national health system policies, the varying prevalence of risk factors between countries and regions, and disparities in human development and the effective delivery of cancer control measures. Strategies to reduce the extent of the disease burden on the continent evidently need to be established locally, to reflect the profile of the observed cancer rates in each European country and in comparison with the pattern in other countries. Approximately, 3.45 million new cases and 1.75 million deaths from

cancer were estimated in Europe in 2012. In combination, cancers of the female breast, colorectal, prostate and lung represent almost half of the overall burden of cancer in Europe. The same diseases are also major causes of cancer death in Europe in 2012, with stomach and pancreatic cancer ranking 4th and 5th respectively, ahead of prostate cancer.

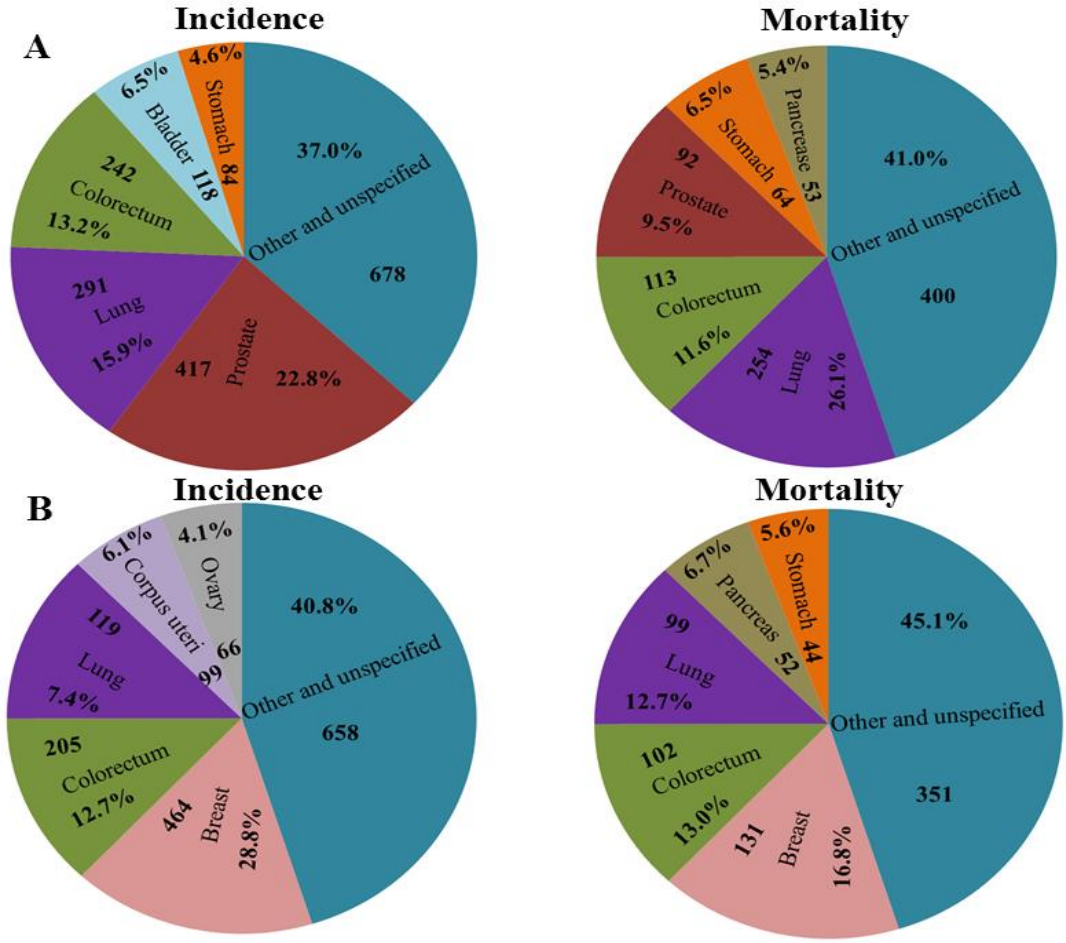

Figure 4: Distribution of the expected cases and deaths for the 5 most common cancers in Europe 2012 in males (A) and females (B). For each sex, the area of the segment of the pie chart reflects the proportion of the total number of cases or deaths [8].

	Number of deaths	Share of all deaths			Standardised death rate				
		Total	Males	Females	Total	Males	Females	Persons	Persons
	(number)	(%)			(per 100 000 inhabitants)				
EU-28	1,294,194	26.0	29.3	22.7	265.1	354.6	202.7	80.8	1,026.1
Belgium	27,361	25.2	28.6	21.9	259.5	345.4	200.5	75.0	1,021.1
Bulgaria	18,169	17.5	19.9	14.8	245.9	344.5	174.9	102.9	836.4
Czech Republic	27,108	24.9	27.4	22.3	289.7	389.2	223.5	86.2	1,129.4
Denmark	15,256	29.2	30.8	27.6	301.6	365.5	258.4	75.8	1,233.7
Germany	224,221	25.0	28.3	22.0	256.2	332.6	204.5	75.0	1,004.0
Estonia	3,679	23.9	26.9	21.1	291.1	468.7	207.3	84.6	1,143.7
Ireland	8,721	29.6	30.8	28.3	286.2	347.4	244.1	70.4	1,176.9
Greece	28,816	25.9	30.6	20.9	250.2	344.3	175.6	76.9	965.7
Spain	107,000	27.5	33.0	21.8	238.9	346.2	160.4	74.1	919.5
France	155,618	27.4	31.4	23.2	245.0	342.0	176.2	80.1	925.5
Croatia	13,803	27.3	31.5	23.2	333.8	477.0	242.5	110.5	1,255.7
Italy	167,648	28.1	32.7	23.8	250.6	339.1	190.0	67.1	1,008.2
Cyprus	1,249	24.2	26.3	21.7	202.3	266.6	151.0	52.4	821.5
Latvia	5,957	20.7	23.5	18.3	300.6	476.1	215.5	105.6	1,105.5
Lithuania	7,838	19.1	21.0	17.1	272.6	424.9	192.2	104.2	967.9
Luxembourg	1,037	27.3	30.4	24.3	243.8	315.8	196.3	68.2	968.8
Hungary	32,733	25.9	28.8	23.0	352.1	487.2	268.1	143.0	1,215.1
Malta	853	26.3	28.3	24.3	230.4	295.0	186.8	58.9	938.5
Netherlands	42,522	30.1	33.8	26.7	284.4	362.4	233.1	77.4	1,138.7
Austria	20,144	25.6	29.0	22.6	249.7	329.8	198.2	70.9	987.8
Poland	94,179	24.3	25.8	22.6	292.4	410.7	219.8	102.4	1,076.9
Portugal	25,899	24.3	28.5	19.9	243.0	350.6	167.4	80.6	913.4
Romania	49,289	20.0	22.6	17.1	269.7	374.5	194.1	118.4	894.5
Slovenia	6,073	31.6	35.7	27.5	314.7	445.2	234.7	91.8	1,234.6
Slovakia	13,444	25.8	28.9	22.5	327.1	476.8	234.4	107.1	1,235.2
Finland	11,896	23.2	24.6	21.7	223.0	288.1	182.0	56.0	912.3
Sweden	22,208	24.7	26.4	23.0	236.8	282.4	207.8	55.4	985.8
United Kingdom	161,473	28.2	30.7	25.8	279.6	342.0	236.1	69.8	1,145.6
Liechtenstein	76	31.3	27.9	34.7	248.3	241.7	254.0	64.5	1,006.8
Norway	10,721	26.7	29.7	23.9	252.9	317.3	210.3	61.6	1,042.5
Switzerland	16,721	25.8	29.6	22.3	223.5	294.2	176.4	57.4	909.3
Serbia	21,108	21.1	23.8	18.2	297.9	391.2	229.0	121.9	1,024.7
Turkey	74,455	20.9	24.9	16.0	186.4	272.5	115.6	70.8	663.6

Table 1: Causes of death — malignant neoplasms, residents, 2013 [34].

Chapter 2: Tumor microenvironment

1. Tumor Microenvironment

Solid tumors harbor not only carcinoma cells but also several noncancerous or stromal cells including fibroblasts/myofibroblasts, epithelial, angiogeneic vascular, lymphatic endothelial, smooth muscle, and immune cells, including T-cells, macrophages, and neutrophils [35]. In many cases, organ-specific interstitial cells are also present, for example, osteoblasts in bone tissue and astrocytes in the central nervous system. Collectively, these cells along with extracellular matrix are termed as tumor stroma and, together with soluble factors, including growth factors, cytokines, oxygen levels, and pH, they make up the tumor microenvironment. Although several steps and factors are involved in tumor progression but the major factor is the bidirectional interaction between tumor cells and their microenvironment [36]. Cancer cells modify and activate their microenvironment while stromal cells along with the extracellular matrix (ECM) also affect cancer cells by secreting soluble factors such as growth factors or cytokines. These interactions can act in an autocrine and a paracrine manner to influence the outcome of the malignancy. The tumor microenvironment provides the signals that activate transcription factors. In order to become established and to grow and spread, tumors need to develop specific capabilities: they must be able to move; remove obstructions to their growth and movement, such as the surrounding extracellular matrix; overcome the immune system and blood-borne cells of the immune system so that they can survive in blood vessels; and establish successfully in their new tissue environment. In order to achieve these functions, tumors recruit host machinery and proteins involved in normal cell growth including transcription factors.

More recently, it has become clear that the tumor microenvironment affects the response to therapies [37, 38]. To prevent tumor progression, it is important to understand the mechanisms governing the relationship between cancer and stromal cells.

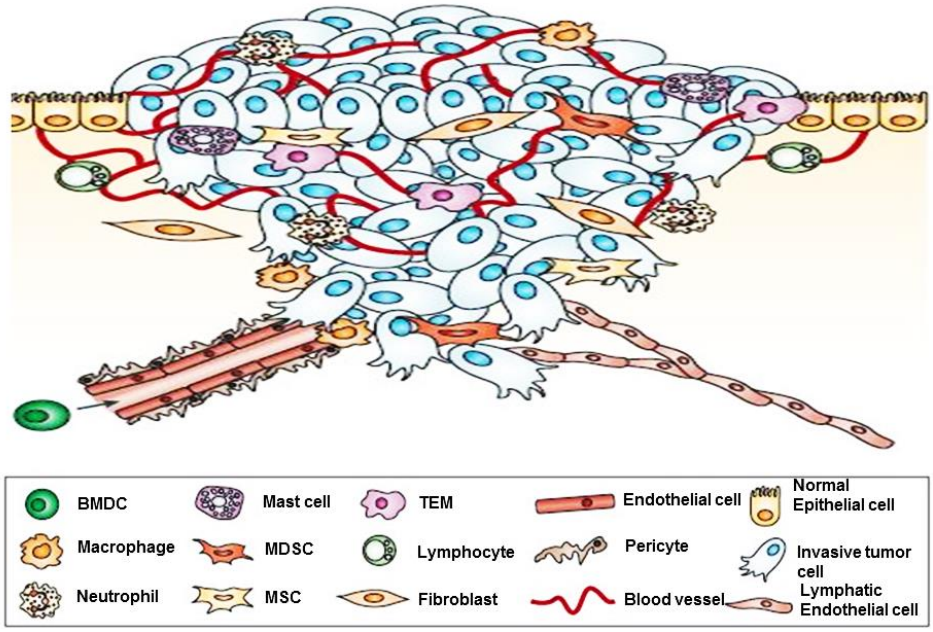

Figure 5: The primary tumor microenvironment. Cancer cells in primary tumors are surrounded by a complex microenvironment comprising numerous cells including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells (BMDCs) including macrophages, myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs) and mesenchymal stem cells (MSCs).

The tumor microenvironment can be categorized into three groups: cells of hematopoietic origin, cells of mesenchymal origin and non-cellular components. Tumors of different origins and different stages of progression contain components in various proportions.

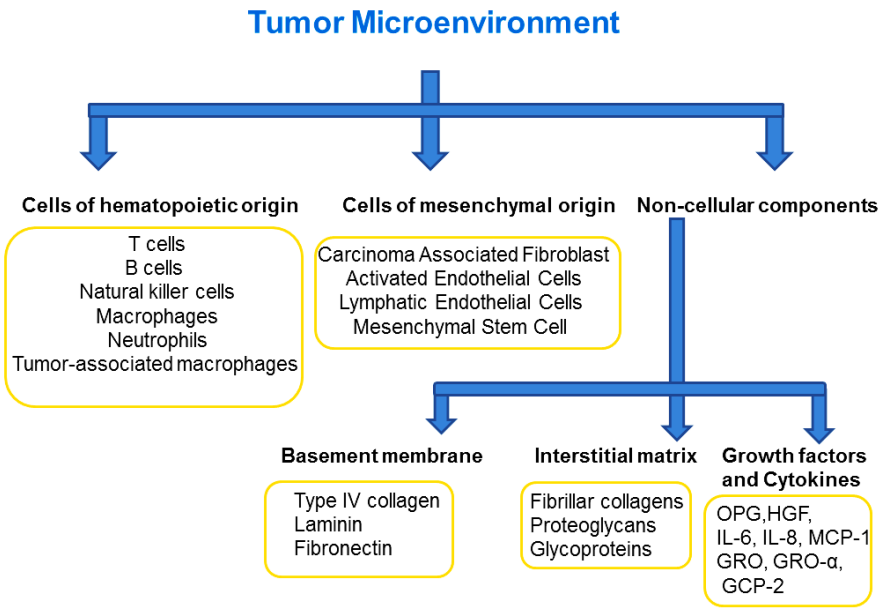

Figure 6: Tumor microenvironment classifications

1.1 Cells of hematopoietic origin

These comprise cells derived from the bone marrow and can be subdivided into cells of the lymphoid lineage, consisting of T cells, B cells and natural killer cells, and those of the myeloid lineage, which includes macrophages, neutrophils and myeloid-derived suppressor cells. The roles of different subsets of T cells in tumor promotion [39] and tumor elimination [40] have been well studied and are the subject of recent advances in the development of novel immunotherapies [41]. Tumor-associated macrophages (TAMs) are believed to be involved in tumor growth, tumor angiogenesis, extravasation of tumor cells, and suppression of antitumor immunity in different types of cancer [42-45]. Ohta et al. found the significant co-relation between macrophage infiltration into tumor tissue and tumor-vessel density of human gastric cancer (GC). Wu et al. [46] also reported the involvement of TAMs tumor-induced angiogenesis and lymphangiogenesis in GC. It is increasingly appreciated that overcoming immune surveillance is a critical part of tumorigenesis [47] and reactivating the process by suppressing “checkpoints” that limit T-cell function is a potent anticancer strategy [48, 49].

1.2 Cells of mesenchymal origin

This compartment consists of cells that arise from the mesenchyme and include fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells. Myofibroblasts and MSCs derived from the bone marrow have been shown to directly support CSCs by creating a favorable niche and helping cancer progression [50].

1.2.1 Carcinoma Associated Fibroblast

Fibroblasts are the major stromal cells within the solid tumor of various types of human carcinomas [51]. Fibroblast recruited around the neoplastic cells, also known as carcinoma-associated fibroblasts (CAFs) or activated fibroblasts, or myofibroblasts [52], form a myofibroblastic microenvironment to promote cancer growth and survival and are characterized by the production of alpha smooth muscle actin (α-SMA). CAFs may be derived from normal fibroblasts, pre-adipocytes, smooth muscle cells, or bone marrow derived cells [53, 54]. The evidence also suggests that the activation of resident fibroblasts is induced by many cancer-secreted factors, such as TGF-β and CXCL12/ SDF-1[55], or by losing suppressor genes, such as PTEN, CAV- 1, p53, and p21 [56-62]. This hypothesis is consistent with breast cancer xenograft models [55]. There is still no evidence that which origin of CAFs is dominant and the same situation is in the markers of them. The most acceptable markers of CAFs include α-SMA, fibroblast-specific protein (FSP-1), fibroblast-activated protein (FAP), platelet-derived growth factor-α receptor (PDGFR-α), PDGFR-β, and vimentin or loss of CAV-1, PTEN, p21, or TP53 mutation [56-62]. However, several studies confirm that the CAF marker is mainly dependent on the tissue origin [63].

CAF-supported microenvironment has a dual tumor developing role. It provides not only the essential signals for tumor cells dedifferentiation, proliferation and survival but also facilitates their local invasion and metastasis [64]. CAFs promote tumor progression in multiple ways such as secretion of multiple factors and matrix metalloproteinases (MMPs), inducing stemness, epithelial mesenchymal transition (EMT), epigenetic changes, etc. [65, 66]. They alter the three dimensional ECM scaffold and support tumor cells that eventually metastasize and stimulate immune cells to augment the ECM-degrading ability [67]. Transforming growth factor β

(TGF-β) induces hepatocyte growth factor (HGF) expression by fibroblasts and also induces the transition of fibroblasts to myofibroblasts by increasing the expression of α-SMA and tenascin C [68]. Rajski et al., reported the gene expression changes induced by insulin-like growth factor-1 in human breast and lung fibroblasts [69], contained several soluble factors, such as periostin which is involved in bone metastasis and angiogenesis [70-72], tenascin, which augments cancer cell proliferation [26].

1.2.2 Activated Endothelial Cells

Over the past few decades, several studies showed that sustained vascularization plays an important role in the growth, invasion and metastasis of cancer [3, 73]. The evidence collected to date suggests the increased number of endothelial progenitor cells (EPCs) in the peripheral blood of cancer patients and the correlation of circulating EPCs with the clinical outcomes [74]. EPCs are a particular subclass of mononuclear cells co-expressing vascular endothelial growth factor receptor 2 and CD34, which could multiply and differentiate into mature endothelial cells [75]. It was reported that cancer cell lines having mesenchymal properties could differentiate blood monocytes into $CD14^{+}/KDR^{+}$ cells via secretion of pleiotrophin and VEGF. These $CD14^{+}/KDR^{+}$ sub-population of cells are endothelial progenitors which exhibit angiogenic property such as formation of tubular structures on matrigel [76, 77]. For the first time, Lyden et al. determined the role of EPCs in angiogenesis [78] for tumor vascularization [79, 80]. Specifically, angiogenesis is a complex process, which comprises of the migration and recruitment of bone marrow derived EPCs to tumor sites and later proliferation and differentiation into mature endothelial cells [81, 82].

It was reported that wild-type mice after bone marrow transplantation had the ability to restore tumor vascularization and growth of numerous kinds of cancer cell lines but not in angiogenesis-defective Id-mutant mice [78]. In subsequent animal xenograft models, 50 % of incorporated EPCs have been found in newly formed blood vessels [83, 84]. Different studies proposed that EPCs might encourage tumor vascularization through an alternative autocrine/paracrine mechanism [85]. Gao et al., demonstrated that angiogenesis could be inhibited by blocking EPCs mobilization and ultimately impaired tumor growth. Moreover, EPCs gene expression could up regulate various pro-angiogenic genes, such as FGFR1, VEGF-C, and platelet-derived growth factor

alpha [86]. Experimental evidence also suggested that an Akt3-dependent VEGF secretion [87] could contribute to the recruitment of EPCs to tumor sites due to an increase in Id-1 expression, an EPC mobilizing factor [88]. In summary, a number of experimental evidences indicate the role of EPCs in tumor vascularization either directly or indirectly.

1.2.3 Lymphatic Endothelial Cells (LECs)

The occurrence of malignant cells in local or sentinel lymph nodes (SLNs) is an indicator of poor prognosis in human cancer [89], which shows the close relationship between lymphatics and cancer biology. It has been reported that peritumoural lymphatic vessels enlargement due to proliferation of lymphatic endothelial cells (LECs) results in greater surface area of potential contact between lymphatic vessels and cancer cells to aid the entry of malignant cells into lymphatics for metastatic spread of cancer [90].

Tumor-induced lymphangiogenesis needs the coordination among several complex cellular events like proliferation, migration, sprouting, and tube formation. Lymphangiogenesis is mediated by lymphangiogenic growth factors that are produced and secreted by the tumors themselves, stromal cells, tumor-infiltrating macrophages, or activated platelets [91-94]. The overexpression of either VEGF- C or VEGF-D in tumors significantly increased tumor-associated lymphatic vessel growth (primarily at the tumor margin) and increased incidence of lymph node metastasis via protein kinase c-dependent activation of the ERK1 or ERK2 signalling cascade and phosphorylation of AKT [95, 96]. The VEGFR3 co-receptor neuropilin 2 (NRP2) also modulates the signalling events that are activated by VEGF-C and VEGF-D [97, 98] . Such signalling pathways are comparable to that involved in angiogenesis by VEGF-A via VEGFR2 and NRP1 [99]. Moreover, the cellular events that characterize angiogenesis like tip and stalk cell differentiation might be similar in lymphangiogenesis.

The identification of the chemokine receptors CXCR4 and CCR7 and the expression of their respective ligands (CXCL12 [SDF-1] and CCL21) on human breast cancer cells that tumors normally metastasize to [100] have led to a rapid expansion in the field of chemotactic tumor metastasis.

After the recognition of importance of lymphangiogenesis and lymphatic remodeling in metastatic spread of carcinoma, the idea to block them by targeting lymphangiogenic signalling pathways, might be a useful therapeutic approach to prevent metastasis [101].

1.2.4 Mesenchymal Stem Cell (MSCs)

According to the International Society for Cellular Therapy, a mesenchymal stem cell (MSC) is defined by its phenotype: CD14 or CD11b, CD19 or CD79, CD34 CD45, HLA-DR, CD73, CD90, CD105, its ability to adhere with plastic, and to be differentiated into three lineages, chondrocyte, osteoblast, and adipocyte [102].

MSCs can favor tumor growth. Fetal or adult MSCs along with tumor cells injected the mice subcutaneously can increase tumor growth [103]. Immunosuppressive property of MSCs could be one explanation for tumor growth enhancement because they modulate immune cells function when activated by a mitogenic signal [104]. It is reported that melanoma cells injected subcutaneously (s.c) into an allogenic recipient could produce tumor only in the presence of MSCs [105]. Interestingly, MSCs coinjected whether at the same site as tumor cells or at distance have the capability to produce tumor into mice [106]. So far, few studies have addressed the identification molecules involved in cancer cell proliferation by MSCs. It has been reported that MSCs increase tumor cell proliferation when coculture (indirect interaction) with breast cancer cells in vitro, which favors the involvement of soluble factors in this phenomenon [107]. The inhibitory effect of MSCs on B-lymphocytes was reported through cell cycle arrest at G_0 /G_1 phase [104]. MSCs are not sensitive to cytotoxic T-lymphocyte (CTL)-mediated lysis and can inhibit CTL cytotoxicity in a dose-dependent manner [108, 109]. Although MSCs do not activate natural killer (NK) cells [109] but can block the action of interferon (IFN) produced by IL-2-stimulated NK cells [110]. The immunosuppressive effect of MSCs is mediated by soluble factors present in the medium when co-cultures them with immune cells but these soluble factors are not clearly identified yet.

MSCs express several proangiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin-1, and growth factors such as fibroblast growth factor 2 (FGF-2), FGF-7, platelet-derived growth factor and cytokines (TNFα, IL-6) [111, 112]. All these molecules promote vasculogenesis and angiogenesis by acting on

endothelial cells synergistically. It has been reported that MSCs activate endothelial cells not only by soluble factors but also by cell contacts between the two types of cells.

The studies about the involvement of MSCs in metastasis are few in numbers. One of the study by Karnoub et al., showed that MSCs could increase the metastasis rate of breast cancer cells through secretion of Rantes (CCL5) by MSCs, suggesting that the main adverse role of MSCs was its pro-invasive potential [113]. This study leads to the participation of other molecules in the augmentation of metastasis by MSCs, and will be a challenge for future studies. Among the different peritoneal stromal cells, hospicells are also a keystone for cancer development through their contribution in the establishment of the pre-metastatic niche and the induction of metastatic and chemoresistant phenotypes [114-116].

1.2.5 Hospicells

It has been reported that mesothelial cells play an important role in the metastasis of gastric, pancreatic and ovarian cancer [117]. Recent findings suggested that mesothelial cells become hemispheric and exfoliate into the peritoneal cavity before implanting to peritoneum. Mesothelial cells closely attached to cancer cells play an active role in tumorigenesis and metastasis [118]. We have isolated mesothelial cells, mentioned as hospicells from the ascites of patients with ovarian cancer and later inside ovarian tumors [114]. These cells are carcinoma associated mesenchymal stem cells that not only promote tumor growth via angiogenesis, epithelial mesenchymal transition, immune suppression and oncological trogocytosis but also induce therapeutic resistance [115]. We have revealed that hospicells were confined around the tumor nodules and adjacent to neovessels suggesting a role in angiogenesis [19].

1.2.5.1 Origin and markers of Hospicells

A key unsolved question on hospicells is their possible multiple origin within the tumor microenvironment. The study demonstrates that hospicells did not express lineage specific cell surface markers such as cytokeratin and EMA (Epithelial Membrane Antigen) specific for epithelial cell lines, vimentin specific for mesenchymal cell lines, CD45 specific for hematopoietic cells, such as granulocytes, monocytes, and B and T lymphocytes, CD20 specific for B-lymphocytes, CD3

specific for T cells, CD34 specific for stem cells of the bone marrow, CD68 specific for macrophages and histiocytes, S100 protein specific for melanocytes and myeloperoxidase specific for granulocytic lineage.

Morphologically hospicells were identified as large cells with pseudopods and can be expanded in vitro or cryopreserved. The surface marker expression profile of hospicells is somewhat similar to MSCs such as CD9, CD10, CD29, CD146, CD166 and HLA-1 but different in the sense that they are negative for other typical markers vimentin, CD73, CD90 and CD106. Gene expression profile of hospicells were found positive for VEGF-A, FGF-2, IL-6, IL-8, TGFβ, MMP2, TNF-1, IGF-1, IGF-2, IGFR1, IGFR2 and TNFα and negative for HGF-1, CCL5, or MMP1 and 9 [119].

The evidence collected to date suggests that hospicells might be derived from MSCs within the tumor microenvironment under the effect of tumor derived factors [113, 120, 121]. These cells have been isolated from the differentiation of stem cells or mononuclear cells characterized by expression of the marker $CD34^+$ and / or $CD133^+$ in the bone marrow or from the cells of an effusion of a patient with cancer [114, 116]. CD146 (melanoma cell adhesion molecule), expressed on hospicells is used as a marker for endothelial cell lineage. MSCs expressing this marker look to be related to perivascular cells of blood vessels [122].

1.2.5.2 Hospicells in the regulation of angiogenesis

It has become evident that we cannot understand tumor growth without considering components of the stromal microenvironment, such as the vasculature. Much research is now devoted to determining the impact of angiogenesis on tumor development and progression, and the reciprocal influences of tumor products on the microvasculature. Using a co-implantation ovarian cancer xenograft model, we demonstrated that the hospicells could promote tumorigenicity in vivo by their action on angiogenesis [119]. This effect on angiogenesis could be attributed to the increased HIF1α and VEGF expression associated with the presence of the hospicells [123, 124]. Recent data suggested an increased expression of HIF-1α in xenografts found by either subcutaneous or intraperitoneal injections of a mixture of cancer cells and hospicells compared with those found following injection of equal volume of tumor cells. This HIF-1α overexpression represented a prognostic factor of aggressiveness of ovarian

tumor [125-127]. Evidence also exists that cancer cells starts binding on the hospicells and secrete growth factors like VEGF, HIFα and cytokines like IL-6, IL-8 for angiogenesis that is an important step for their growth. However the involvement of these growth factors, cytokines and chemokines in the presence of hospicells are still under consideration to explain the role of the hospicells in angiogenesis.

Angiogenesis inhibition is a promising strategy for cancer therapy. No current significant results were obtained in the treatment of ovarian cancer by using antiangiogenic targeting VEGF, key mediator of angiogenesis, such as bevacizumab (monoclonal antibodies directed against VEGF) or cediranib (inhibitory VEGF receptor molecule) [128]. Therefore, other proangiogenic factors including chemokines must also be included to improve anti-angiogenic strategies — not only for cancer treatment, but also for preventing recurrence.

1.2.5.3 Hospicells associated chemoresistance to chemotherapy for cancer cells via oncological trogocytosis

Mesothelial cells are the most important allies of cancer cells for the acquisition of chemoresistance in peritoneum. Recent findings showed the involvement of hospicells in the acquisition of chemoresistance of ovarian tumors via trogocytosis. This ‘‘trogocytosis’’ between tumor cells and neighboring mesothelial cells is an important step for the induction of chemoresistance of previously sensitive cells. Several mechanisms can take place during intercellular communication such as link through ligand receptor connections, molecules transporting through gap- junctions and lastly relocation of surface membranes proteins convening new properties to the tolerant cells [129-131]. The importance of contact between the hospicells and cancer cells closes out the role of secreted factors in the development of ovarian tumor chemoresistance. This collaboration induced independent achievement of chemoresistance and the existence of hospicells might have consequences on patients’ prognoses. Recent study showed the existence of MDR (functional P-glycoprotein) proteins on hospicells from many different patients with a variable expression. Levchenko et al. have described intercellular transfer of multidrug resistance protein (MDR) proteins among several tumor cell lines [132]. Specificity, the role of intercellular communication, and their morphology revealed by electronic microscopic analysis suggested the existence of ‘‘oncologic synapses’’ as compared

to ‘‘immunological synapses’’. This transfer of MDR protein from hospicells to ovarian tumor cells for chemoresistance acquisition advocates the importance of hospicells in the progress or control of neoplastic disease. The finding of cellular mechanisms and their pathways leading to intercellular recognition and ‘‘oncologic trogocytosis’’ with hospicells could be helpful to identify novel therapeutic targets for cancer therapy.

1.2.5.4 Hospicells in metastasis of cancer cells

Metastasis, the spread of tumor cells to tissues and organs far from where the original tumor created and the development of new tumors, is the sole event leading to death of most cancer patients [133]. This process is generally related to epithelial-mesenchymal transition (EMT) encouraged by a number of soluble factors secreted either by the cancer cells themselves or by cells involved in cancer microenvironment. Hospicells not only encourage tumorigenesis but also support invasion and metastasis in breast and ovarian carcinoma. It has been reported that breast cancer cell line (MDA-MB-231) along with hospicells acquires membrane patches through oncologic trogocytosis [134]. This oncologlical trogocytosis is an active unidirectional process depending on actin polymerization which can be increased or decreased by the inhibition of the Src family and phosphoinositide 3-kinase (PI3K) respectively. Cell-cell attachment is directly related to PI3K signaling pathway and its inhibition disrupts cell-cell adhesion in both mammary and intestinal epithelial cells resulting in decreased trogocytosis [135, 136].

The acquisition of N-cadherin in hospicells is an important metastatic phenotypic property of cancer cells especially breast cancer cells which is not directly related to trogocytosis between MDA-MB-231 and hospicells but through the production of various soluble factors. [137, 138]. Among these soluble molecules, one candidate could be collagen I secreted by hospicells due to its capability for encouraging the up-regulation of N-cadherin in human pancreatic cancer cells [139] and mouse mammary epithelial cells [140]. Recent data showed the expression of TGF-ß in hospicells via gene profile [119] which could promote metastasis in ovarian tumor via increased matrix metalloproteinase secretion or increased expression of N-cadherin [141]. It has been reported that human lung cancer cells increases production of TGF-ß in response

to collagen I, which in turn support EMT changes through the expression of N-cadherin [142].

Consideration of acquisition of metastatic characteristics by the cancer cell through interactions with hospicells could be supportive to identify novel therapeutic strategies for cancer.

1.5.5.5 Cross talk between hospicells and immune cells within tumor microenvironment

Tumor immunologists have revealed many cellular and molecular mechanisms which facilitate tumor escape from natural immune surveillance and acts as extrinsic tumor suppressor [143, 144]. Within the cancer microenvironment, immune cells not only shut down their anti-cancer immune response effectively, but also interact closely with the transformed cells to stimulate oncogenesis. The immunomodulatory role of hospicells might intervene with immunity to tumor. It has been reported that hospicells inhibit the proliferative capacity of immune cells ($CD4^+$, $CD8^+$ and $V\gamma9V\varrho2$ T cells) and their secretions of cytokines in vitro without direct contact [145]. This immunosuppression is mainly due to nitric oxide formed by the inducible nitric oxide synthase and to the tryptophan degradation products by indoleamine 2,3-dioxygenase (IDO). NO inhibits the activation of proteins by blocking phosphorylation in the IL-2 receptor-signaling cascade, including Janus kinases 1 and 3, STAT5 (signal transducer and activator of transcription 5), Erk and Akt [146, 147] which might inhibit T-cell proliferation by hospicells. Recent experimental data showed that the accumulation of $FoxP3^+$ regulatory T cells in the ascites of ovarian cancer patients suppress tumor-specific T-cell immunity leading to decreased patient survival [148]. Many soluble immunosuppressive factors including PGE 2, TGF-β or IDO by-products secreted from stromal cells within the microenvironment might encourage transformation of effector $CD4^+$ T cells into $FoxP3^+$ regulatory T cells [149-151]. Hospicells might either convert effector T lymphocytes into regulatory T lymphocytes or contribute to the accumulation of regulatory T lymphocytes in cancer patients remains to be determined.

We proposed that immunosuppression of T lymphocytes mediated by hospicells in tumor microenvironment permit cancer cells to evade immune surveillance. Thus to

recover the immune response for effective chemotherapy against carcinoma, targeting of hospicells could be helpful.

1.3 Non-cellular components

The major non-cellular component of the tumor microenvironment is the extracellular matrix (ECM). The ECM includes the basement membrane and the interstitial matrix. The basement membrane is rich in type IV collagen, laminin and fibronectin and the interstitial matrix, which consists of fibrillar collagens, proteoglycans and glycoproteins that contribute to the tensile strength of the tissue [152]. The ECM provides not only a physical scaffold for all cells in the TME but also has a dynamic role in the evolution and metastasis of cancers, especially as the adhesion of cancer cells to the ECM is a key to their movement into and out of the TME. The ECM also contains key growth factors, such as angiogenic factors and chemokines, that interact with cell surface receptors and give each tissue its tensile and compressive strength and elasticity [153]. The ECM is involved in the formation of a stem cell niche and although it acts to maintain tissue architecture and prevent cancer cell invasion, an abnormal ECM has been reported to promote cancer progression and angiogenesis [152]. Matrix metalloproteases (MMPs) that degrade ECM proteins are secreted and activated by cancer cells, TAMs and CAFs. MMPs further remodel the ECM, thereby also secreting chemokines and growth and angiogenic factors.

Chapter 3: Peritoneal carcinomatosis

1. Peritoneal cavity

The peritoneal cavity is a potential space between the parietal peritoneum (the peritoneum that surrounds the abdominal wall) and visceral peritoneum (the peritoneum that surrounds the internal organs) [154, 155]. Both the parietal and visceral peritonea are not different but the same peritoneum given two names depending on their function/location. The peritoneal cavity contains a large amount of adipose tissue and is covered by a mesothelium, which has a smooth and non-adhesive surface that facilitates intra-coelomic movement. In addition to its unique anatomical structure, the peritoneal cavity contains many types of immune cells, such as lymphocytes, macrophages, and granulocytes, and mesothelial cells, which contribute to direct cell-cell contacts between tumor cells.

2. Mesothelial cells

The mesothelium consists of a monolayer of specialized cells (mesothelial cells) which extends over the entire surface of the three serosal cavities (pleural, pericardial and peritoneal) and the organs contained within these cavities. The primary function of the mesothelium is to provide non-adhesive surface barrier against physical damage and invading organisms and a frictionless interface for the free movement of opposing organs and tissues. However, mesothelium is also involved in inflammation, tissue repair, coagulation, fibrinolysis and tumor cell adhesion [156].

Mesothelial cells are derived from the mesoderm but express both mesenchymal and epithelial cell intermediate filaments. Mesothelial cells rest on a thin basement membrane supported by connective tissue stroma. The cells are predominantly flattened, squamous-like, approximately 25µm in diameter, with the cytoplasm raised over a central round or oval nucleus.

Mesothelial regeneration involves migration of cells from the wound edge and attachment and incorporation of free-floating mesothelial cells from the serosal fluid onto the wound surface. Impaired healing leads to the formation of fibrous serosal adhesions. Mesothelial cells have the ability to change their phenotype comparable to changes seen in epithelial-to-mesenchymal transition (EMT). Mesothelial cells undergo EMT during continuous ambulatory peritoneal dialysis (CAPD), with the

induction of the transcription factor *snail* and a dramatic down regulation of E-cadherin [157].

The exact role of mesothelial cells in tumor cell adhesion and growth is unclear. Many studies have demonstrated that traumatised mesothelial surfaces are privileged sites for tumor cell adhesion [158] possibly due to upregulation of adhesion molecules on mesothelial cells in response to inflammatory mediators and exposure of underlying extracellular matrix (ECM). They can also regulate ECM turnover by secreting matrix metalloproteinases and tissue inhibitors of metalloproteinases.

Mesothelial cells also play an important role in local fibrin deposition and clearance within serosal cavities. Their fibrinolytic activity is a key factor in the prevention and removal of fibrin deposits that form following mechanical injury, hemothoraces and infection. If the fibrinolytic capacity is insufficient and fibrin accumulation is not resolved, fibrous adhesions form between opposing serosal surfaces [159]. Mesothelial cells have both procoagulant and fibrinolytic activity [159]. The procoagulant activity is due to tissue factor, the main cellular initiator of the extrinsic coagulation cascade.

3. Peritoneal Carcinomatosis

Several gastrointestinal and gynecological malignancies have the potential to disseminate and grow in the peritoneal cavity. This condition is often associated with disease progression and poor prognosis. Peritoneal carcinomatosis (PC) is a condition characterized by a multistep phenomenon, including the detachment of cancer cells from the primary tumor, freeing of the cells from the peritoneal cavity, and subsequent attachment to the peritoneum [160]. The process of attachment of tumor cells to the mesothelial cells of the peritoneum involves neoangiogenesis and is mediated by several growth factors. Angiogenesis is essential for oncogenesis but also the viability and expansion of ovarian cancer to any organ or structure that is covered by the peritoneum. Specifically, VEGF is involved in the formation of ascites and has a direct effect on ascites tumor cells as well as an immunosuppressive function [161].

PC frequently occurs in recurrent abdominal malignancies, such as stomach, colorectal and ovarian cancers. Overall survival in patients with PC is generally only slightly influenced by systemic chemotherapy, so that the occurrence of PC is

traditionally regarded by the surgeon as a terminal condition. In 10-35% of patients with recurrent colorectal cancer and in up to 50% of patients with recurrent gastric cancer, tumor recurrence is confined to the peritoneal cavity: those patients have been shown to ultimately die from complications of locoregional tumoral widespread, in most cases without occurrence of metastases in other sites. PC is the most frequent type of metastasis in patients with gastric signet ring cell adenocarcinoma (diffusely infiltrating carcinoma) [162]. They appear in the terminal stage and significantly worsen the prognosis of this type of gastric carcinoma [163]. Despite the importance of peritoneal metastasis in mediating the mortality of gastric carcinoma, little is known about the mechanism of this phenomenon [164]. However penetration of the gastric serosa and lymphatic spread are the two most important factors affecting prognosis in gastric cancer [202-204].

Multimodal approaches combining aggressive cytoreductive surgery, intraperitoneal hyperthermic chemotherapy and systemic chemotherapy have been proposed and are actually considered as promising methods to improve loco-regional control of the disease and ultimately to increase survival [165, 166]. In case of ovarian cancer, there is general agreement that complete removal of peritoneal seedings is associated with longer survival, in colorectal and gastric cancer complete removal of peritoneal carcinomatosis is usually followed by short-term recurrence, so that patients are usually treated with limited palliative resection or gastrointestinal bypass without the intent for complete cytoreduction.

Peritoneal carcinomatosis is a real challenge for oncologists and surgeons, which treatment is very difficult. Many surgeons and oncologists are still use to raise the white flag in discovering them.

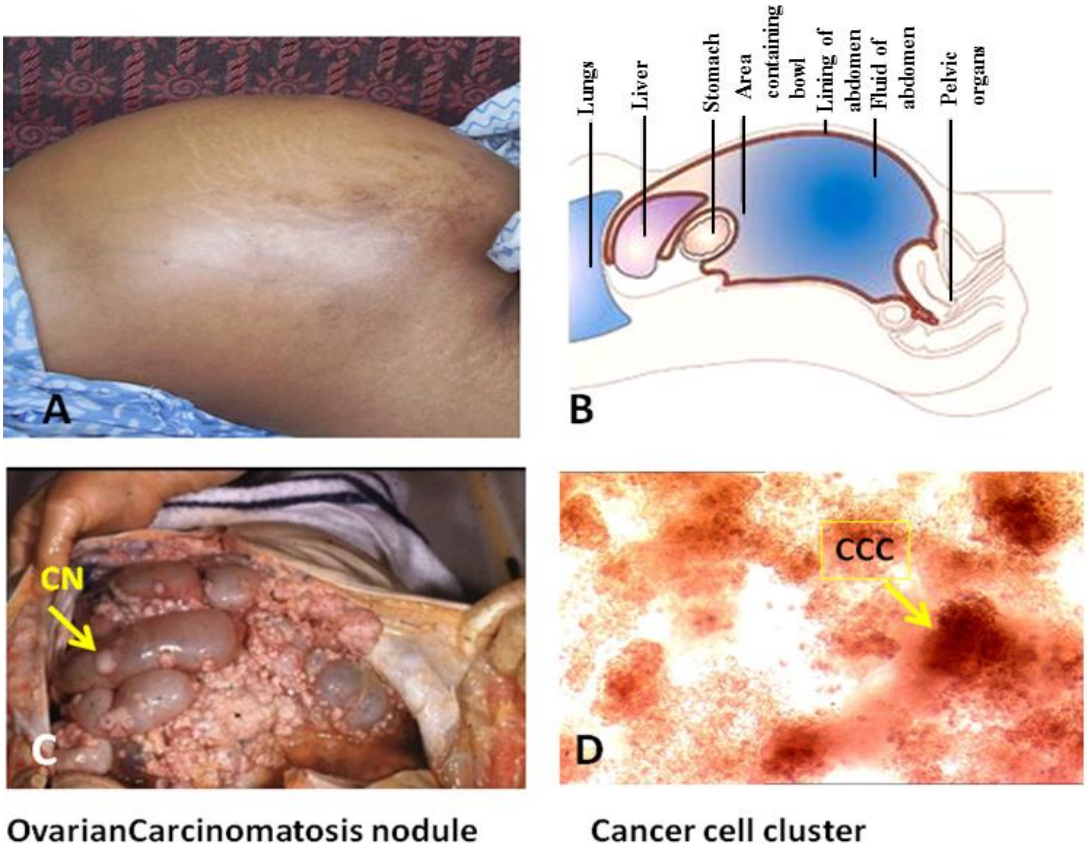

Figure 7: Peritoneal metastasis from ovarian origin. Abnormal accumulation of abdominal fluid, ascites (A, B) having carcinomatous nodules (C) and floating cancer cell cluster/spheroids (D)

4. Molecular Biology of Peritoneal Carcinomatosis

Peritoneal carcinomatosis refers to the complex sequence of events by which tumor cells spread from their primary organ of origin to establish independent metastatic deposits on the visceral and parietal peritoneal lining of the abdominal cavity. With few exceptions, once peritoneal dissemination occurs the malignant process is deemed non-curative as it is seldom amenable to surgical resection and current chemotherapeutic regimens are merely palliative. An understanding of the molecular events involved in peritoneal carcinomatosis is therefore of paramount importance if we are to advance therapeutic strategies for this devastating form of cancer progression [167].

1.5 Peritoneal Tumor Dissemination

It is well recognized that viable tumor cells can be isolated from ascitic fluid or by direct contact with the tumor at the time of surgery and their presence has been linked with poor prognosis [168, 169]. In a similar manner, perforation of the primary cancer, which may either be spontaneous or occur inadvertently during surgery, increases the rate of local recurrence and reduces survival [170-172]. Alternatively, tumor cells may be inadvertently liberated from transected lymphatics and blood vessels during the course of surgical resection. Whatever the mechanism of spillage, once liberated from their normal tissue constraints, the tumor cells are free to be disseminated around the peritoneal cavity [173].

1.6 Mesothelial invasion

Before invading tumor cells can gain firm adherence to the submesothelial connective tissue, they must penetrate the mesothelial monolayer. Two possible mechanisms exist: either tumor cells invade the intercellular spaces between adjacent mesothelial cells; or they must destroy the mesothelial monolayer [174]. However, other researchers have commented on a change in mesothelial morphology that occurs in areas of tumor cell invasion [163, 175]. *Iwanicki MP, et al,* found cancer spheroids attached to the mesothelial monolayer using various cell adhesion molecules promotes dissociation of mesothelial cells adhesions and their migration away from intruding tumor cells, leading to mesothelial cells exclusion from the base of cancer spheroid [176]. Yonemura et al. explored this observation further using a mouse model and the gastric cell line, MKN-45-P [177]. Intraperitoneal inoculation of MKN-45-P resulted in mesothelial contraction and eventual exfoliation. Similar effects could be induced in vivo by intra-peritoneal injection of IL-6, TNF-β and IL-8, and in-vitro by cytokine stimulation of mesothelial monolayers. It was postulated that tumor-derived cytokines were responsible for disruption of the mesothelial barrier, exposing the submesothelial basement membrane, and facilitating tumor adhesion. These studies suggested that mesothelial cells retracted in the presence of the tumor. Furthermore, the cancer cells did not adhere to the mesothelial cells, but rather to connective tissue under the mesothelial cells. In addition, electron micrographs of excised human peritoneum–associated tumors revealed that mesothelial cells are not present directly under the tumor mass, suggesting mesothelial clearance from the area

beneath the tumor mass [178]. Early, in vitro experiments also provided evidence that mesothelial cells retract after coming in contact with tumor cells [163, 179]. In these studies, ovarian cancer cell clusters disrupted mesothelial cell–cell junctions and penetrated under mesothelial cells, suggesting that the integrity of the mesothelial cell monolayer is altered by the attached tumor cells that bind with high affinity to the submesothelial matrix [163, 180].

Mesothelial cells undergo a transition from an epithelial phenotype to a mesenchymal phenotype with loss of epithelial morphology and decrease in the expression of cytokeratin and E-cadherin when exposed to conditioned medium from HSC-39, and the induction of mesothelial cells can be abolished using a neutralizing antibody to transforming growth factor-beta1 (TGF-β1) as well as by pre-treatment with SB431542 [167].

1.7 Stromal invasion and proliferation

Having attached to the peritoneum and penetrated the mesothelial barrier, tumor cells must next gain stable adherence to the submesothelial connective tissue before they can invade and proliferate. Current evidence suggests that the connective tissue growth factor induced EMT of human peritoneal mesothelial cells is associated with an increased adhesion of gastric cancer cells to mesothelial cells, suggesting that tissue factor promotes gastric cancer cell adhesion to peritoneum, which facilitates malignant cell dissemination [181]. Schlaeppi et al. found that adhesion of colorectal cell lines to extracellular matrix components was completely integrin dependent [182].

Further proliferation and survival of the adherent tumor cells requires a compatible interaction between the invading cells and the peritoneal stroma. Although the consequences of tumor-stromal interaction have been much studied in other metastatic systems, this interaction has received little attention with respect to peritoneal metastasis development. Assuming tumour cells successfully attach to the submesothelial connective tissue and have encountered a favorable host response, it is then necessary for them to invade the extracellular matrix. The matrix metalloproteinases (MMPs) may play a central role in stromal invasion. Yonemura et al. studied the role of metalloprotease in a mouse model of peritoneal carcinomatosis [183].

Chapter 4: Epithelial Mesenchymal Transition and Chemoresistance

1. Epithelial Mesenchymal Transition

The most comprehensive theory describing how initially quiescent tumor cells acquire metastatic capability is the epithelial-mesenchymal transition (EMT). EMT has become progressively better characterized in the last five to ten years and has emerged as a primary theory of how tumor cells acquire the characteristics necessary to metastasize. The phenomenon whereby epithelial cells can lose their epithelial characteristics and acquire mesenchymal characteristics was first described in the early 1980s [184]. Phenotypically, cells become more spindle-shaped and lose basal-apical polarity [185]. They become mobile and plastic in shape and acquire apoptosis resistance and stem like characteristics. This dramatic cell transposition process, known as the epithelial mesenchymal transition (EMT), not only plays critical roles in governing embryonic development and maintaining adult tissue hemostasis (e.g., via regulating wound healing and stem cell behavior, but also contributes to many pathological conditions, such as fibrosis and cancer progression [186-189]. Its molecular hallmark is the down regulation of the cell-cell adhesion molecule E-cadherin, resulting in dissolution of cell-cell tight junctions, and up-regulation of a number of mesenchymal markers, including vimentin, α-SMA and fibronectin [190]. Moreover, several key molecules such as Snail, Slug, matrix metallo proteinases (MMPs) have also been involved in the EMT process in ovarian tumor progression [187, 191, 192].

E-cadherin is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues[193]. Reduced expression of E-cadherin has been observed in aggressive tumors of the esophagus, ovary, stomach and breast [194-198]. Mechanisms by which E-cadherin protein expression is lost include E-cadherin gene mutation and loss of the wild-type allele by loss of heterozygosity [199-201]. These data indicate that E-cadherin is a classic tumor suppressor gene [199, 202].

Vimentin is one of the most widely expressed and highly conserved proteins of the type III intermediate filament protein family. Vimentin has gained much importance as a canonical marker of EMT, a cellular reprogramming process in which the epithelial cells acquire a mesenchymal phenotype that renders the cells to dramatically alter their shape and exhibit increased motility [203]. Increased vimentin

expression has been reported in various tumor cell lines and tissues including prostate cancer, breast cancer, endometrial cancer, CNS tumors, malignant melanoma and gastrointestinal tumors including pancreatic, colorectal and hepatic cancers [204]. In gastric cancers, vimentin expression was more associated with the invasive phenotype of gastric carcinoma and was suggested to play an important role in the metastasis of gastric carcinomas and serve as a prognostic marker in the detection of gastric cancers [205, 206].

α-SMA is known to contribute to cell-generated mechanical tension and maintenance of cell shape and movement. As cell motility is critically dependent on the actin cytoskeleton, the dynamics of cytoskeletal structures affected by α-*SMA* could be essential to invasion and metastasis in lung adenocarcinoma [207, 208]. The microvascular density was significantly associated with VEGF and α-SMA expression and related to tumor location, tumor size and histological degree of differentiation [209].

Fibronectin is one of the major structural components of the basement membrane [210]. Altered expression of fibronectin, degradation and organization have been associated with a number of pathologies, including cancer [211, 212]. Several of the morphological changes observed in tumor types and tumor-derived cell lines have been attributed to decreased expression of fibronectin, increased fibronectin degradation, and/or decreased expression of fibronectin-binding receptors, including α5β1 integrins [213, 214]. Fibronectin is also a candidate biomarker for several solid tumor types, including ovarian, prostate, breast and gastric cancer [210, 215, 216]. In addition to its implication in cancer development, fibronectin also acts as a potential biomarker for treatment-associated resistance [217].

Snail is a typical transcription factor that could induce epithelial-mesenchymal transition (EMT) and cancer progression partly by suppressing the expression of E-cadherin. Reduced expression of E-cadherin may lead to the loss of cell-cell adhesion and cancer progression [218]. In recent years, snail was found to be highly expressed in several carcinomas, including non-small cell lung carcinomas, ovarian carcinomas, urothelial carcinomas, breast cancer, and hepatocellular carcinoma [219-223]. Studies of immunohistochemical analyses suggest that snail is highly expressed in gastric cancer and significantly associated with tumor progression and metastasis [224-226].

Slug is a member of the snail family of repressors, and is expressed in the neural crest and mesodermal cells emigrating from the primitive streak in chick embryos [227]. Recently, another critical role of Slug has been reported. Slug binds to E-box elements in the proximal E-cadherin promoter and represses transcription of the E-cadherin gene [228, 229]. Some reports have found that Slug plays a role in tumor progression in primary human cancers. Castro Alves et al. [230, 231] reported the mRNA expression of Slug and E-cadherin in gastric cancer.

Type I collagen, found in most connective tissue and embryonic tissue [232], is an important member of the collagen family which is a key structural component of the extracellular matrix. Recently, similar to the expressions of other members of the collagen family which is believed to be involved in carcinogenesis [233-235], abnormal expression of type I collagen has also been reported in several cancers [236-238].

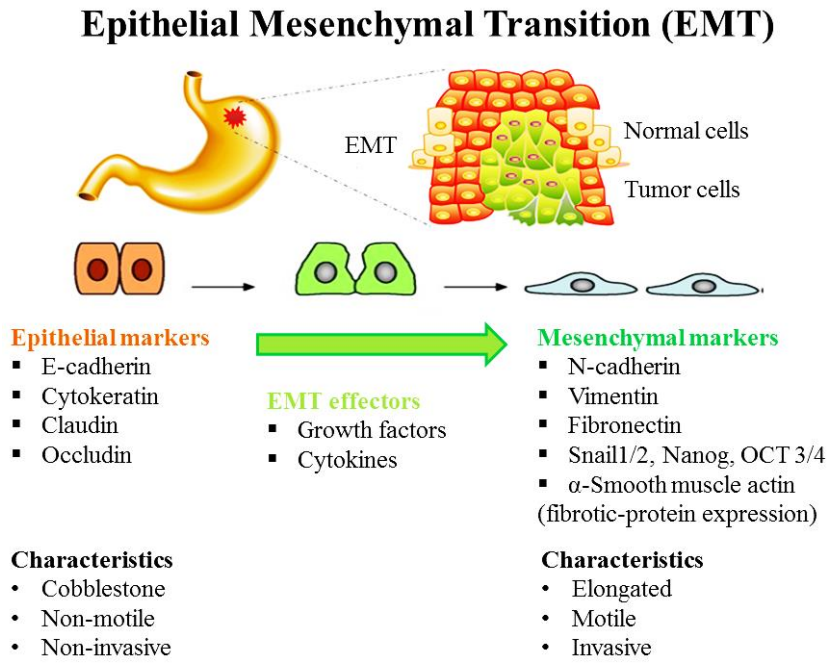

Figure 8: Characteristics of epithelial-mesenchymal transition.
Gastric cancer cells are capable of undergoing epithelial-to-mesenchymal transition under certain culture conditions. Cancer cells express epithelial markers such as E-cadherin, cytokeratin, and tight junction proteins and mesenchymal markers such as N-cadherin, vimentin, fibronectin, Snail, Slug, Twist, NANOG, and OCT3/4.

2. EMT-related signaling networks that regulate E-Cadherin

Most signaling pathways implicated in the start of EMT result in the down-regulation of E- cadherin, that acts as the gatekeeper of EMT [239]. This decreased functional E-cadherin leads to the loss of cell adhesion, resulting in rapid cell growth and motility [239]. E-cadherin also acts as a tumor suppressor protein, preventing cells from rapid cell growth and division in an uncontrolled way; decreased E-cadherin has been associated with metastasis and invasion in murine models of mammary, gastric, prostate, and pancreatic cancer [240]. Providing additional correlative support, E-cadherin is highly down-regulated in many diffuse-type carcinomas such as gastric cancer and lobular breast cancer, in which cells in a solid tumor lose epithelial characteristics and show a highly invasive EMT-derived histological pattern.

Molecular events during EMT result in transcriptional regulation of the transcription factors such as Snail, Slug, and Twist that acts as a phenotypic marker during EMT [241]. β-Catenin not only interacts with E-cadherin to maintain cell–cell adhesion but is also shuttled to the nucleus where Wnt acts as transcription effector of the Wnt signaling pathway to regulate slug activity and link EMT with cancer [242]. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumor cells [243]. Snail is induced by growth factors such as HGF, FGF and EGF via PI3K-Akt or Ras-MAPK pathway. In response to signal from EMT inducer like TGF-β, Snail factors are induced with the help of Smads [244]. Hypoxia-inducible factor-1 regulated lysyl oxidase is involved in an EMT process by interacting with Snail to downregulate E-cadherin expression in epithelial cells and supporting their implication in tumor progression. Tumor necrosis factor-α-induced tumor initiation and metastasis are mediated by the activation of NF-kβ-, PKCα- and AP-1 dependent pathways. Nuclear factor-kB is critical for TNF-α induced tumor promotion.

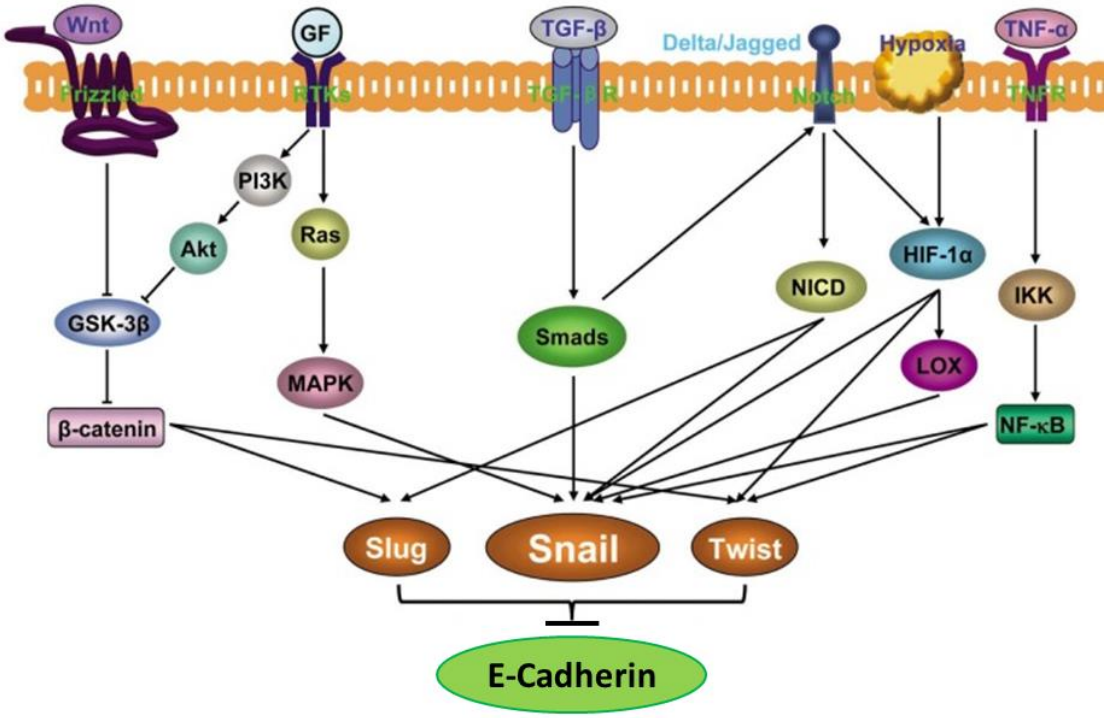

Figure 9: Microenvironmental signaling pathways negatively regulate E-cadherin expression

3. Chemoresistance

The current research that improves the knowledge about drug resistance development is based mainly on drug sensitive and resistant cancer cell lines.

Although chemotherapy remains one of the primary therapeutic modalities used in the treatment of gastric cancer, chemoresistance limits the effectiveness of chemotherapy and results in treatment failure. A wide range of molecular mechanisms of chemoresistance has been implicated in gastric cancer, including reduced intracellular concentrations of drugs and alterations of drug targets. The dysregulation of cell survival and death signaling pathways can also lead to resistance to chemotherapeutic drugs. In addition, the interaction between cancer cells and the tumor microenvironment also plays an important role in chemoresistance whether intrinsic or acquired, in gastric cancer [245]. However, the mechanisms of chemoresistance are complex and multifactorial. The chemotherapeutic resistance of tumors may be caused by different molecular mechanisms in different patients due to tumor heterogeneity and drug variety. Therefore, more extensive studies are needed for a

more comprehensive elucidation of the mechanisms of chemotherapy resistance in gastric cancer.

One of the main reasons of low effectiveness of chemotherapy in cancer patients is drug resistance, which is inherent or, more often, acquired during treatment [246]. In most cases drug resistance has features of Multiple Drug Resistance (MDR). MDR is designated as an insensitivity of cancer cells not only to previously used drug but also to many other drugs with different chemical structure and mechanism of action [247]. Majority of drugs used in chemotherapy act as cytotoxic agents then as cytostatic ones. Although cancer cells develop various mechanisms of resistance to cytotoxic drugs the first players implicated in MDR are drug transporters from ABC family. These proteins use energy from ATP hydrolysis for active removing drugs from the cancer cells [248]. The most important drug transporter is glycoprotein P (P-gp) encoded by the multidrug resistance protein 1 gene (MDR1, ABCB1) [249]. Expression of this protein was noted in over 50 % of cancers with MDR phenotype and it can be inherent or induced by chemotherapy [250]. Approximately 20 different cytotoxic drugs are substrates to P-gp [251] and two of them - paclitaxel [252] and doxorubicin [253] - are commonly used in chemotherapy of many cancers. The second most important drug transporter is breast cancer resistant protein (BCRP) encoded by ABCG2 gene, cloned for the first time from breast cancer cell line MCF-7 [254]. The upregulated expression of BCRP was noted in many cancers including breast [255] and ovarian [256] and is known to protect cancer cells against mitoxantrone [255, 257] and topotecan [256, 257]. Other important ABC transporters implicated in MDR of cancers include MRP1 and MRP2 (MDR1-related protein 1 and MDR-related protein 2) encoded by ABCC1 and ABCC2 genes, respectively [248, 258, 259]. Substrates used by MRP1 are similar to those for P-gp with the exception of taxanes [6]. Among many MRP2 substrates the most important is cisplatin (CIS) and it is the most frequently used antitumor agent in cancer therapy [251, 257].

Another protein involved in MDR, but not belonging to ABC drug transporters family, is MVP/LRP major vault protein/lung resistance - related protein [260]. The upregulation of MVP/LRP expression was noted in lung cancer and was correlated with poor response to chemotherapy [261]. LRP expression increased after exposure to CIS in non-small-cell lung cancer cells [262].

Human P-glycoprotein is encoded by the multidrug resistance 1 (MDR1) gene and is widely expressed on the plasma membrane, Golgi membrane, and intracellular canaliculus of normal human tissues, including the liver, kidney, colon, adrenal gland, intestine, placenta, hematopoietic precursor cells, and endothelial cells at the blood-brain, blood-placenta, and blood-testis barriers. P-Glycoprotein functions in the transport and/or secretion of its substrates and the protection of these tissues from physiologically active substances, cytotoxic agents, and xenobiotics [263-265]. P-Glycoprotein expression is significantly elevated in drug-resistant tumors and pumps out various anticancer drugs, such as anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes [266-268]. Thus, P-glycoprotein expression in cancer cells confers MDR to these anticancer drugs.

Lung resistance-related protein (LRP) is a further protein related to MDR. LRP was first detected in a non- P-glycoprotein-mediated multidrug-resistant lung cancer cell line [269] and has been shown to be the major human vault protein [270]. A predictive or prognostic value of LRP expression has previously been reported for ovarian cancers (8), diffuse large B-cell lymphomas [271] and adult T-cell leukaemias [272]. Fan et al. [273] investigated the LRP expression in gastric cancers and stated that LRP expression was not significantly related to clinicopathological factors.

With the development of modern biological techniques, the mechanisms of chemoresistance have been broadly investigated in recent years. It is important to focus on the molecular mechanisms of chemoresistance in gastric cancer and on recent studies that have sought to overcome the underlying mechanisms of chemoresistance.

Molecular Mechanism of Chemoresistance		Ref.
Reduced Intracellular Concentration of Drugs		
Drug Efflux	The expression of MDR1 is associated with chemoresistance in cancer patients and has been found in human gastric cancer cell line.	[274, 275]
Drug Inactivation	The overexpression of glutathione S-transferase (GST-pi) has been found to be significantly related to the sensitivity of gastric cancer to cisplatin.	[276]
Reduced prodrug activation	Impaired activity enzymes like thymidine phosphorylase, uridine phosphorylase and orotate phosphoribosyl transferase has been associated with chemoresistance to 5-FU in gastric cancer.	[277-279]
Alteration in Drug Targets		
DNA topoisomerases	A reduction in topoisomerase-II expression was found to contribute to the resistance of human gastric cancer cells to topoisomerase-II -targeted drugs like Adriamycin.	[280]
Dysregulation of Cell survival and Death		
BCL-2 family members	The silencing of BCL-2 decreases resistance to 5-FU in gastric adenocarcinoma cells.	[281-283]
p53	The restoration of p53 has been found	[284, 285]

	effective to overcome cisplatin resistance in gastric cancer by metabolic alteration.	
PI3K/AKT pathway	Activated AKT is associated with increased resistance 5-FU, adriamycin, mitomycin C, cis-platinum and paclitaxel.	[286-288]
Mitogen-activated protein kinase pathway	The activation of the p38-MAPK pathway was found to be responsible for the modulation of multidrug resistance in gastric cancer	[289]
Tumor Microenvironment		
Hypoxia	HIF-1α expression could predict the response of patients with advanced gastric cancer to 5-FU-based adjuvant chemotherapy.	[290, 291]
Alterations of the extracellular matrix	Laminin could modify the response to chemotherapeutic agents by regulating of MDR-related proteins (ABCB1 and ABCC1).	[292, 293]
Cytokines and growth factors	IL-6 and Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) could be associated with acquisition of resistance of gastric cancer cells to trastuzumab and 5-FU respectively.	[294, 295]

Table 2: Mechanism of chemoresistance of tumor cells

4. EMT-associated resistance to cancer therapeutics

Cancer therapy is often associated with two major forms of drug resistance—de novo or acquired. Patients who are initially refractory to therapy show intrinsic or 'de novo' drug resistance. Patients that initially respond to therapy typically relapse as a consequence of 'acquired' drug resistance.

More recently, evidence has shown that drug resistance and EMT are very closely correlated. Several studies have demonstrated that EMT widely exists in breast cancer [296], gastric cancer [297] colon cancer [298], ovarian cancer [299], and in a fraction of bladder cancer patients [300]. Increased evidence has demonstrated that EMT renders tumor cells more resistant to chemotherapeutic drugs when cells are transfected with some hallmark genes of EMT, including Notch, Twist, and TGFβ [296, 301, 302]. All of these data led to the hypothesis that tumor cells would become more resistant after they had undergone EMT. However, other reports have demonstrated that it is the emergence of EMT in MDR cells that inhibits the effect of chemotherapeutic agents. In reality, both sides have indicated that EMT plays an important role in the process of drug-mediated resistance. During the early stages of tumor development, ABC proteins have long been considered a major target of EMT and a potential contributor to drug resistance. The relationship between EMT and ABC transporters is still not clear, but there is considerable evidence linking the presence of binding sites in ABC transporter promoters with EMT-inducing transcription factors [303, 304]. Saxenad et al. [305] demonstrated that down-regulation of EMT-inducing transcription factors could reduce the expression of ABC transporters and render drug resistant cells more chemosensitive. Furthermore, they found that ABC transporters carry several binding sites for EMT-inducing transcription factors, including Twist, Snail, and FOXC2, which directly regulate ABC transporters and increase their promoter activity.

Chapter 5: Cancer stem cell differentiation

1. Cancer stem cells

Stem cells have the unlimited ability to self-renew and the capacity to differentiate into several specialized cell types. Solid tumors contain phenotypically and functionally heterogeneous cancer cells known as cancer stem cells (CSCs). Several researchers have focused on the understanding of CSCs after the existence of CSCs in leukemia was described in 1994 [12]. CSCs display characteristics that are similar to normal stem cells, including unlimited self-renewal, proliferation, and multi-lineage differentiation. It is also postulated that CSCs occupy the same niche, called the CSC niche, as normal stem cells [306].

Recent studies demonstrated that CSCs are responsible for tumor initiation, invasion, distant metastasis, and resistance to anticancer drugs, so therapies that target CSCs are becoming increasingly appealing [307]. Currently, CSCs have been found in many types of solid tumors, such as breast cancer [308], gastric cancer [309], ovarian cancer [310] and colon cancer [311].

Two formal possibilities remained: either all solid cancer cells had a low probability of proliferating extensively and behaving as cancer stem cells or most cancer cells have only a limited proliferative potential and cannot behave as cancer stem cells, but a small, definable subset of cells is enriched for the ability to proliferate extensively and form tumors. In both cases, some of the cancer cell heterogeneity would arise as a result of environmental differences within the tumor and continuing mutagenesis. The essential difference between these possibilities is the prediction, according to the second possibility, that whatever the environment or mutational status of the cells, only a small, phenotypically distinct subset of cancer cells has the ability to proliferate extensively or form a new tumors (Fig). It has not been possible to distinguish between these models of solid cancer heterogeneity, because as yet no one has published the identity of purified subsets of uncultured solid cancer cells that are enriched for the ability to form new tumors [312].

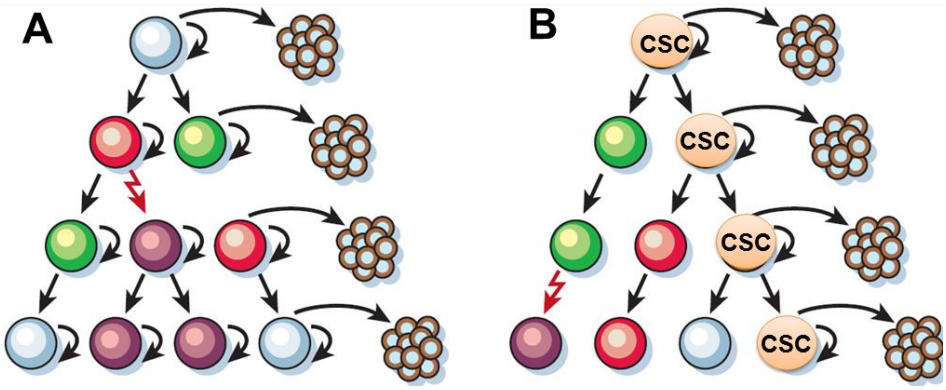

Figure 10: Two general models of heterogeneity in solid cancer cells.
Tumor cells are heterogeneous, but most cells can proliferate extensively and form new tumors **(A)**, Tumor cells are heterogeneous and only the cancer stem cell subset (CSC; yellow) has the ability to proliferate extensively and form new tumors **(B)**.

CSCs are one of the promising areas of cancer research. It is essential to understand the biology of CSC to develop a strategy to eradicate cancer. In various cases, malignant tumor tissues include CSCs, which overcome various anticancer therapies targeting specific proteins despite the temporary effect against the progression of cancer. Given that numerous genes are involved in cellular processes in CSC cell cycles, researchers are yet struggling to find promising cancer therapies.

Several gene sets have been identified to have the potential to transform cancer cells into CSCs or maintain the homeostasis of CSCs. For example, epithelial to mesenchymal transition (EMT) inducers are representative functional markers for the acquisition of a stem-like state in cancer cells [313]. ATP-binding cassette (ABC) transporter proteins in CSC-like cells function to secure genomic stability and prevent apoptosis by efflux of cytotoxic agents [314]. A variety of developmental signaling pathways, such as the Wnt, Notch, Hedgehog, BMP, FGF, IGF, and TGFβ pathways, are known to affect stem cell self-renewal and differentiation [315]. It is important to understand the highly complicated signaling mechanism, which sustains the CSC biology.

Inhibiting one of the important pathways for CSC causes the activation of the bypass pathway; therefore, some researchers are seeking a different approach. This approach originates from the ideas of differentiation, which causes leukocyte-initiating cells (LIC) to differentiate into terminally differentiated leukocytes [316, 317]. The induction of differentiation has expanded to solid tumors [318].

1.1 Identification of cancer stem cells within gastrointestinal tumor

Different methods have typically been used to identify CSCs in published studies [319, 320]. One is an in vitro technique include "spheroid colony formation or cell surface marker expression," and in vivo approach is to implant CSCs under the skin or within organspecific sites (e.g., orthotropic) of immunodeficient mice. Some stem cell surface markers expression are given below

Marker	Characteristics	Ref.
CD44	CD44(+) gastric cancer cells demonstrated properties of chemo- and radio-resistance	[321]
CD73	CD73 expression correlates closely with HIF-1α expression in gastric carcinoma	[322]
CD90	CD90 phenotype is enriched in sphere-cultured cells from human gastric primary tumors	[323]
CD105	CD105 expressed in gastric carcinoma are correlated to vascular invasion, distance metastasis and the development of ascites.	[324]
CD117	Immunostaining with CD117 used for diagnosis and prognostic classification of gastric cancer	[325]
CD133	CD133 (+) colon cancer cells are associated with AKT and increased radiation resistance	[326]
CXCR-4	CXCR4 positivity of primary gastric carcinomas significantly correlated with the development of peritoneal carcinomatosis	[327]
NANOG	NANOG a molecular markers to reflect the differentiation status of gastric cancer	[328]
OCT3/4	OCT3/4 expression is associated with stemness tumor	[329]

	cells in human gastric cancer	
SOX-2	Sox2-positive expression is associated with invasion of gastric cancer	[330]
DNMT-3B	DNMT-3B expression in cells is associated with gastric cancer development	[331]

Table 3: Cancer stem cell markers

1.2 Key signaling pathway in CSCs

The maintenance of stem cell nature partly depends on the regulation of several signaling pathways. The pathways and signal molecules related to the control of CSCs self-renewal, differentiation and apoptosis include PI3K/Akt, PTEN, JAK/STAT, Wnt/β-catenin, hedgehog, Notch, and NF-kB [332-340].

The activation of signal molecule Akt plays a critical role for cellular transformation and tumorigenesis. The PI3K/Akt signaling pathway regulated the growth of many cancers. Guo et al.'s study revealed that Akt1 enhanced the resistance to apoptosis by increasing the expression level of Bcl-2 and the phosphorylation of the pro-apoptotic protein BAD [336].

The tumor generation related signaling pathway JAK/STAT was activated at the same time. To control the suppressor of cytokine signaling-3 (SCS-3) is also observed as an effective way to regulate tumor development by activating JAK/STAT signaling pathway [341]. The Notch signaling pathways have also been shown to be involved in tumor growth, metastatic initiation and self-renewal processes. The over expression of Notch3 could lead to the expansion of CSCs and chemoresistance enhancement in ovarian cancer. The sensitivity of CSCs was clearly increased to platinum therapy after inhibiting the Notch signaling pathway via siRNA knockout. In normal stem tissues, Hedgehog and Wnt feedback is associated with the proliferation of epithelial stem cells [342]. It is well known that stem cell compartments exist within stem cell niches, and these signaling pathways are important for stem cell niches in tissue homeostasis [343, 344]. The Hedgehog signaling pathway plays an important role in tumor initiation, metastasis, and maintenance of cancer stem cells [345, 346]. In cancer cells, Wnt/β-catenin signaling has been regarded as an important pathway for

upregulating EMT-associated gene expression [347, 348]. In addition, the well-known hedgehog and Wnt signaling pathways also play fundamental roles in maintaining CSC populations [349].

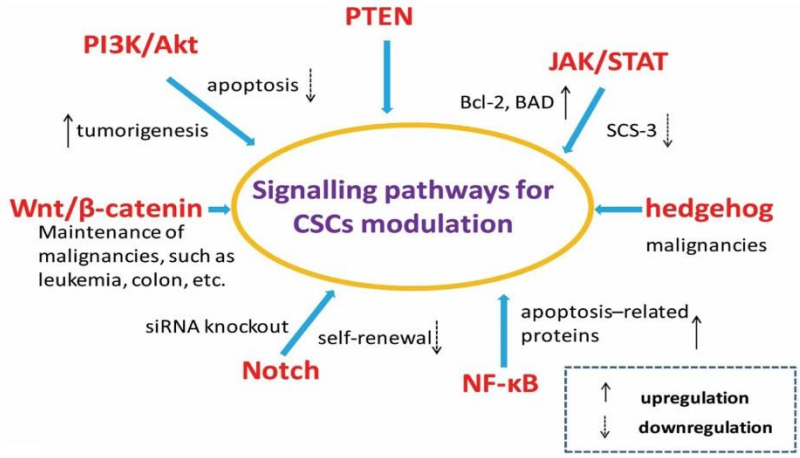

Figure 11: Signal pathways related with cancer stem cells.
Pathways and elements involved in the control of self-renewing and differentiation of cancer stem cells as well as normal stem cells include PI3K/Akt, JAK/STAT, Wnt/β-catenin, hedgehog, Notch and NF-κB.

1.3 Emerging role of microRNAs in cancer stem cells

The most important progress over the past recent years is the discovery of miRNAs that are small (19–25 nucleotides long) non-coding single-stranded RNAs [350]. They control gene expression by targeting mRNA transcripts and leading to their translational repression or degradation, according to the level of complementarity with them [3, 4]. This degradation includes decapping, deadenylation and exonucleolytic cleavage of the target mRNA transcript [351-353]. More than 2,700 potential human miRNAs have been recorded in miRBase v20 [354] and their number is increasing rapidly. Taking into consideration that one miRNA can target many mRNA transcripts and that one mRNA transcript can be targeted by several miRNAs, it can be estimated that 10–40% of the mRNA sequences are targeted by miRNAs in human [355].

Several essential roles of miRNAs include the regulation of cell development, differentiation, stress response, apoptosis, and proliferation by regulating the stability or translational efficiency of target messenger RNAs [356, 357]. MicroRNAs also act as novel oncogenes or tumor-suppressor genes. Reports have demonstrated that miRNAs regulate the development of CSCs and the acquisition of the EMT phenotype [358, 359]. The discovery of miRNAs has complex molecular networks

regulating EMT and stemness in cancer metastasis [358, 360]. Thus, microRNAs might be promising therapeutic targets for tumor [361, 362].

The role of miR-200 and miR-205 in EMT and tumor progression has been related to numerous cancers by targeting ZEB1 and SIP1 [244, 363-365]. Valladares-Ayerbes, Manuel, et al. found circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer [366]. The miR-200 expression was also demonstrated to change the tumor microenvironment and inhibit EMT and metastasis, in lung adenocarcinoma [367]. Gregory et al. revealed low miR-200 levels in cells that had undergone EMT, in response to TGF-β. The enforced miR-200 expression alone was also reported to be enough to check TGF-β-induced EMT and miR-200 inhibition was sufficient to induce EMT. miR-205, located in 1q32.2, was shown to be overexpressed in head and neck cancer compared with other cancer lines from lung, breast, colorectal, prostate, and pancreas [368]. Upregulation of miR-205 was observed in bladder cancer [369] as well as in adult mouse corneal and footpad epithelium [370].

miR-21 is a tumor-associated miRNA for which aberrant expression was first identified in glioblastomas [371]. miR-21, located in 17q23.2, has been shown to be upregulated in breast cancer [372, 373], gastric cancer [374], lung cancer [357], and glioblastoma [375]. Antisense studies have shown that it controls cell growth by inhibiting apoptosis [371] but does not affect cell proliferation. The notion that miR-21 functions as an oncogene was very recently supported by showing that suppression of miR-21 can inhibit tumor growth through downregulation of tumor suppressor tropomyosin I [376]. Many human let-7 genes map to regions altered or deleted in human tumors [377], indicating that these genes may function as tumor suppressors. In fact, let-7g maps to 3p21, which has been implicated in the initiation and progression of lung cancers [378, 379]. Ohshima, Keiichi, et al. reported that let-7 miRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line [380].

1.4 EMT-induced formation of CSCs

Recent studies indicate that metastatic cancer cells, which have presumably undergone EMT, may exhibit a CSC phenotype [381]. This EMT-induced stemness endows cancer stem cells with the ability to self-renew, and to contribute in tumor recurrence, metastasis, and drug resistance [382]. EMT has been confirmed to play a

critical role in tumor metastasis and recurrence, which have been shown to be tightly linked with the function of CSCs [383-386]. Reports have demonstrated that cells undergoing EMT can acquire stem cell-like characteristics, which indicated an interesting conjunction between EMT and stem cells [387, 388].

The signaling pathway links between EMT and the gain of CSC properties are still not explicit; however, the formation of EMT and CSCs has been shown to be a dynamic process, and it is triggered by multiple cellular signaling pathways, such as TGFβ, Wnt/β-catenin, Hedgehog, Notch, and others [389-392]. Recent evidence suggests that the expression of certain genes involved in CSCs are influenced by transcription factors of EMT (Twist, Snail, Slug, Zeb1, SIP1), implicating EMT as potential factors involved in stem cell maintenance. The link between EMT regulators and CSCs points to CSCs as the molecular and cellular explanation for the relationship between EMT and cancer metastasis [393]. In summary, compelling evidence exists relating EMT to the emergence of a CSC-like phenotype, which may be prerequisites for cancer cell metastasis. Furthermore, EMT may provide a new perspective for cancer stem cell theory as well as stem cell research in the relevant area of tumor formation.

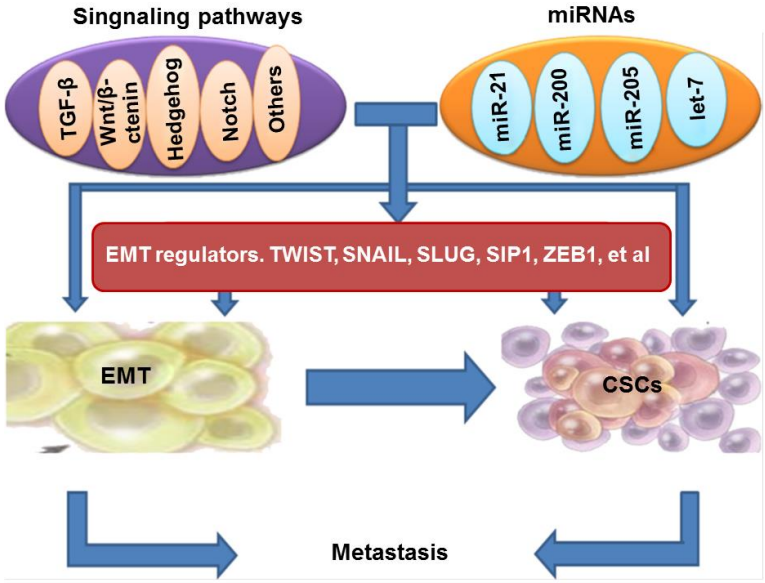

Figure 12: Signaling pathways linking EMT and CSCs.

1.5 Tolerance against chemotherapy in cancer stem cells

CSCs can survive in severe conditions, although most cancer cells die under these conditions. Shutdown of an important signaling pathway for cancer cells can reduce bulk tumor; however, CSCs survive by releasing various agents to the extracellular environment. The expression level of the ATP-binding cassette (ABC) transporters gene such as ABCB1, which encodes P-glycoprotein, and the half-transporter ABCG2, which was originally identified in mitoxantrone-resistant cells correlates with normal cells and stem cell compartment characterized by SP. These transporters were originally found in hematopoietic stem cells by the side population (SP) assay [394, 395]. *MDR1* gene knockout mice were viable and fertile, but showed an increased sensitivity to drugs [396], suggesting that the ABC transporter is important to protect cancer stem cells from chemotherapeutic agents [397]. However, the transporter is not critical for stem cell growth. BCRP is described as an essential multidrug transporter against mitoxantrone, doxorubicin, and daunorubicin in breast cancer [254]. To date, the subfamily of ABC transporter genes has been identified, including the ABCA, ABCB, ABCC, and ABCG subfamilies, and play an important role in the efflux of the vast number of anticancer drugs [398]. In melanoma, anticancer drug treatment with vemurafenib and dacarbazine resulted in the selection of ABCB5-expressing cells [399]. The nuclear membrane is important for securing genomic stability. ABCC2 is localized to the nuclear membrane [400] and involved in resistance to several types of chemotherapeutic agents [401-404]. These studies imply that current chemotherapy should focus on certain types of cancer cells; particularly CSCs. Eliminating CSCs from tumor tissue is the most desirable cancer therapy because these cells play important roles in cancer progression.

2. Strategies to eliminate CSCs

With the recent exploration advancing in CSCs, the CSCs targeted therapy has brought a new hope to the cancer patients. The identification and characterization of CSCs have revealed numerous strategies of cancer treatment in-depth via specific molecular therapies [405]: for example, interfering the cell growth microenvironment of a tumor; targeting the specific biomarkers of CSCs; or inhibiting the key signaling pathways to interfere CSCs activity. Strategies of CSC targeting based on their properties were shown in table.

Targeting cancer stem cells		
Mechanism	**Targets**	**Ref.**
Tumor microenvironment	Vascular niche	[406]
	Endothelial cells	[332]
	use IFN-β	[407]
CSCs-dependent signaling pathways	PI3K/Akt	[336, 338]
	JAK/STAT	[408]
	Notch	[337]
	Hedgehog	[409]
CSC-dependent markers	CD44	[410]
	CD73	[411]
	CD90	[412]
	CD105	[413]
	CD117	[414]
	CD133	[415]
	CXCR-4	[334]
Interfering the CSCs differentiation	ATRA inhibitor	[416]
	Vitamin D3 inhibitor	[417]
	dimethylsufoxide inhibitor	[418]
Immunotherapy	vaccines	[419]
	TGF-β inhibitor	[420]
	Blockade of immune checkpoints	[421]
Exploiting metabolic differences	Inhibit the mTOR	[422]
	Inhibit the mevalonate metabolic	[423]

Table 4: Targeting CSC-specific markers or signaling pathways for cancer therapy

3. Differentiation therapy

It is now clear that CSCs and normal stem cells share similar signaling pathways for regulating cell self-renewal and differentiation, and they can be identified using the same markers. Thus, inhibitors of signaling pathways or markers are not selectively toxic against CSCs, but inevitably cause damage to normal stem cells and eventually affect the regeneration of normal tissue instead. Generally, differentiation agents tend to have less toxicity than conventional cancer treatments. It should also be noted that inducing CSCs differentiation alone may fail to cure patients with solid tumors because proliferating cells will be left untouched with enough divisions to kill the patient [424]. Therefore, targeting both EMT pathways and CSC differentiation is a safer therapeutic strategy to potentially disrupt this axis of evil in the war on cancer. This seems to be feasible, since several studies have successfully shown that pharmacological agents can modulate the differentiation state of a tumor.

The differentiation therapy of solid tumors is defined as a therapeutic strategy that allow malignant cells to revert to a more benign phenotype by inducing the re-activation of endogenous differentiation programs, in which the proliferative and metastatic abilities of cells are diminished and the expressions of mature cell markers are restored [425].

The idea of converting malignant cells to benign cells as a possible therapy for patients with cancer was first proposed in 1961 by Pierce, [426] when self-differentiation was observed in teratocarcinomas in his laboratory. In the 1970s and 1980s several pioneering reports popularized the potential of this strategy as a therapeutic protocol for hematological malignancy [427]. However; the transformation of this idea to a real successful clinical practice was not realized until 1984, when the use of all-trans retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL) was reported [428]. ATRA induces terminal cell differentiation by disrupting the promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion protein that arrests the maturation of myeloid cells at the promyelocytic stage [429]. Subsequently, emerging studies have focused on elucidating the mechanisms of action of differentiation therapy in cancers, particularly in solid tumors. In a paper published on *Nature Communications*, Yan et al. [430]

demonstrated that restoring IkB kinase α (IKKα) expression led to cellular differentiation in nasopharyngeal carcinoma (NPC).

This breakthrough has changed this fatal disease into one of the most curable of all forms of leukemia [431] and has greatly expanded studies on the clinical application of differentiation therapy for tumors. Although most of these studies focus on the explorations of hematological malignancies such as leukemia, more and more attention is being paid to differentiation pathways in solid tumors, which might shed new light on differentiation therapy for solid tumors.

As highly differentiated cells rarely divide, and rapidly proliferating cells have poorly differentiated phenotypes, two basic therapeutic approaches for combating cancers might be developed: ‘‘differentiation therapy’’ [432] to induce differentiation and ‘‘destruction therapy’’ [433] to thwart malignant proliferation.

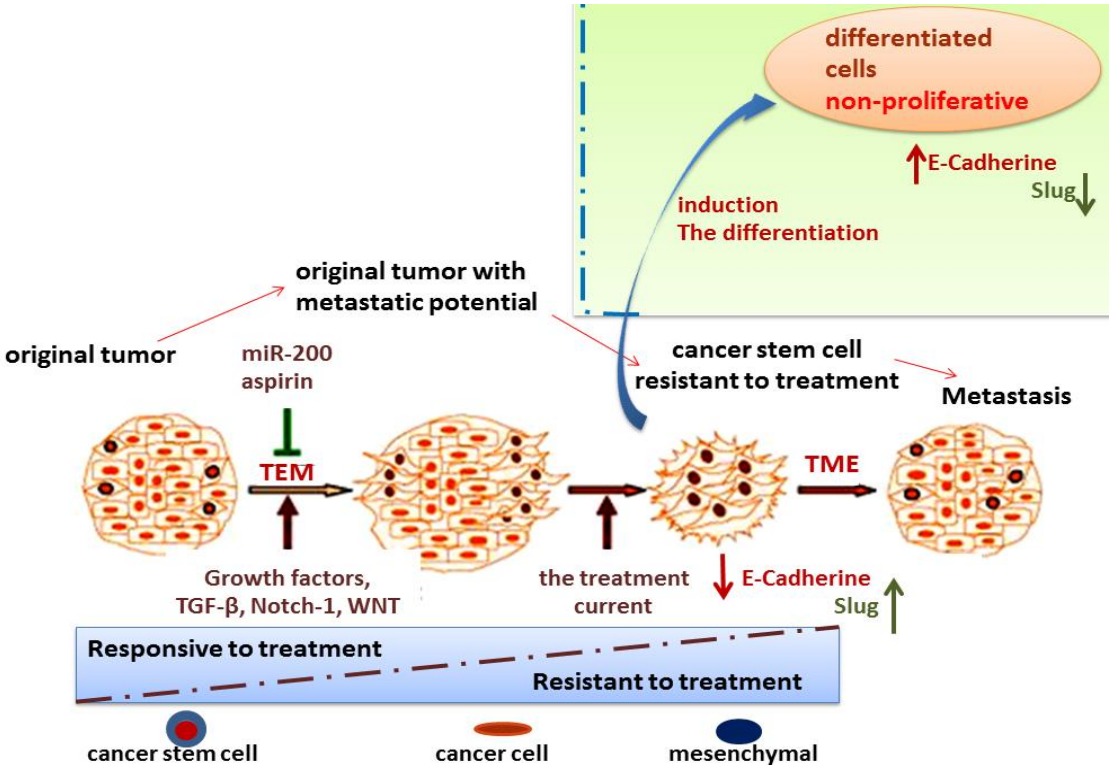

Figure 13: Induction of epithelial-to-mesenchymal transition (EMT)-phenotypic cells produces cancer stem-like cells with drug-resistant characteristics. Growth factors, including FGF, EGF, PDGF-B and PDGF-D as well as factors such as TGF-β, Notch-1 and Wnt, can induce EMT, while miR-200 family inhibits EMT by regulating the expression of transcription repressors ZEB1 and ZEB2. EMT-phenotypic cells acquire stem-like cell signatures characterized by increased metastatic capacity, self-renewal ability and acquired drug resistance. These cells metastasize to distant sites and undergo MET to produce metastatic tumors that are phenotypically similar to the primary tumor.

Chapter 6: Heparanase (HPSE)

1. Heparanase

Mammalian HPSE is the only endoglycosidase capable of cleaving HS chains of HSPGs. It exerts its enzymatic activity by catalyzing the cleavage of the β (1,4)-glycoside bond between glucuronic acid and glucosamine residues, generating fragments of about 5–7 kDa. Human HPSE gene (HPSE1) encodes a polypeptide of 543 amino acids (Pre-proheparanase) and 68 kDa that are synthesized in the endoplasmic reticulum. Cutting of the N-terminal signal peptide gives rise to the inactive latent HPSE precursor of 65 kDa (pro-HPSE), which is readily secreted [434]. Activation of the precursor into the mature enzyme demands the reuptake of the latent protein and intracellular proteolytic processing. Endocytosis is mediated mainly by syndecans and requires the syndecan-interacting proteins syntenin and α-actinin [435]. Once inside the lysosome, the precursor undergoes the cleavage of a linker region (by cathepsin-L), giving rise to 8- and 50-kDa subunits that form the mature dimeric enzyme [436]. Within the lysosome, HPSE is implicated in HSPGs turnover. Outside the cell, HPSE degrades cell surface HS and matrix and promotes ECM remodeling and the release of HS-linked molecules, including growth factors and cytokines. Apart from its primary perinuclear localization within lysosomes and late endosomes, several studies have found mature HPSE in the nucleus during in vitro cancer cell differentiation [437, 438].

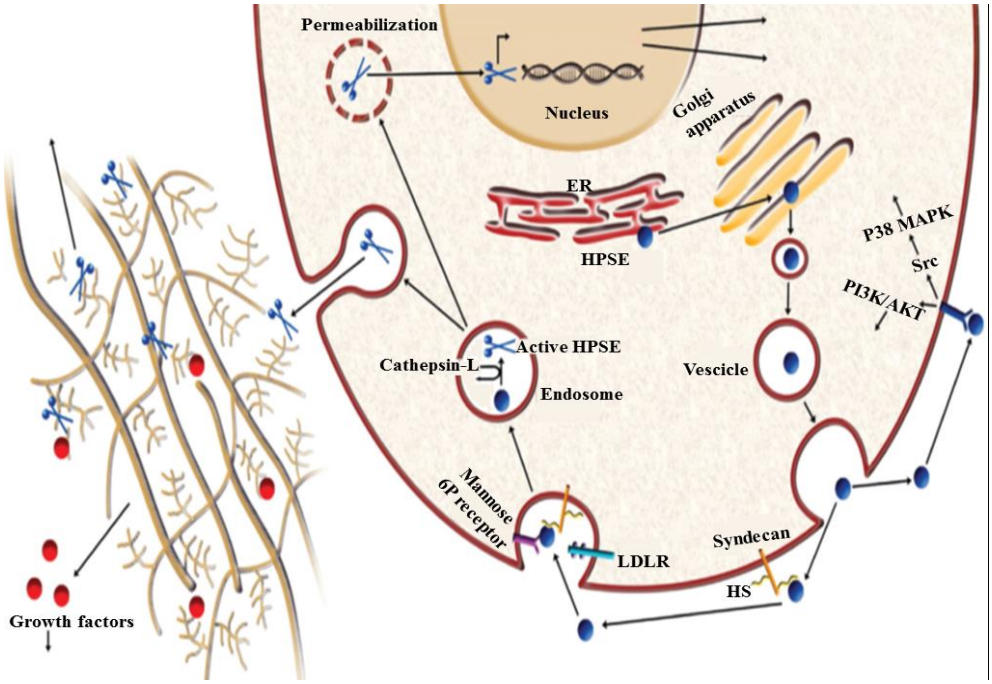

Figure 14: HPSE: processing, localization, enzymatic and non-enzymatic activities. HPSE is synthesized in the endoplasmic reticulum as a latent precursor (pro-HPSE). After moving to the Golgi apparatus, pro-HPSE is secreted outside the cell (1). HPSE uptake [mediated by syndecans, mannose-6-phosphate-receptor and low-density lipoprotein receptor (LDLR)-related protein] (2), and its delivery to lysosome enables the proteolytic processing (by cathepsin-L) and activation of the precursor into the dimeric active enzyme (3). The interaction of latent HPSE with HPSE-binding proteins activates various intracellular signaling pathways implicated in angiogenesis, cel[439]l adhesion and migration (4). HPSE secretion or translocation into the nucleus may occur under the effect of different stimuli. Extracellular HSPGs degradation encourages cell migration, thus enhancing tumor cell invasiveness and metastasis (5). Angiogenesis, EMT and inflammatory response are indirectly regulated by HPSE via HS-linked growth factors that are released after HS cleavage (6). Nuclear HPSE translocation is implicated in cell differentiation, inflammation and glucose metabolism through gene transcription regulation mechanisms (7).

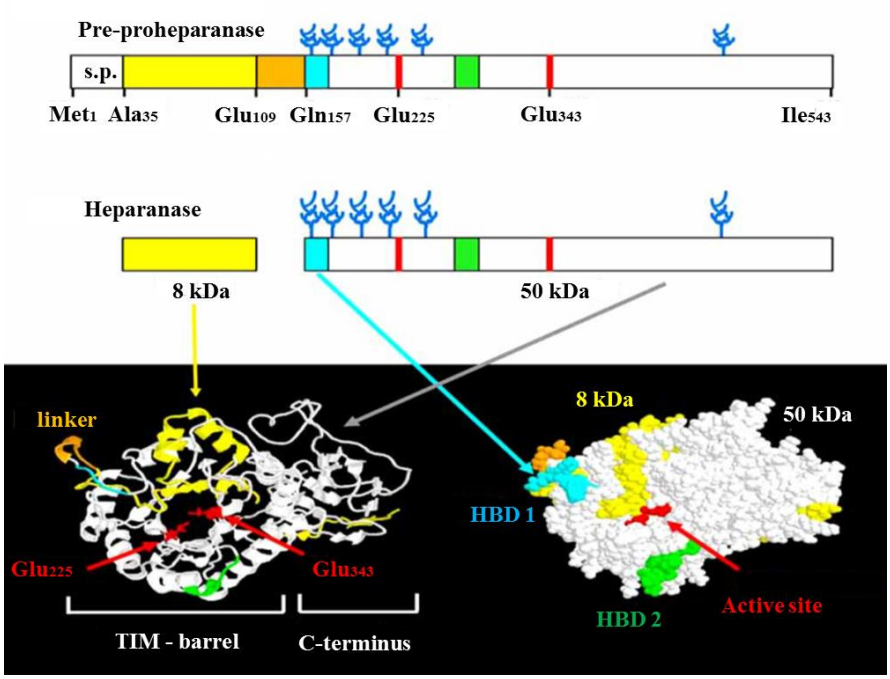

Figure 15: Primary structure, critical amino acids and predicted three-dimensional structure of the heparanase heterodimer. Pre-proheparanase harbors 35 amino acids signal peptide (s.p., Met1-Ala35) which is removed upon entering the ER. The protein is then subjected to glycosylated at six N-glycosylation sites ( ) and secreted as a latent ~65 kDa protein (upper panel). Proteolytic processing removes the linker domain (Ser110–Gln157), resulting in an 8 kDa (Gln36–Glu109) and 50 kDa (Lys158–Ile543) protein subunits (middle panel) that heterodimerize to yield an active enzyme. A predicted three-dimensional structure of the heparanase heterodimer (bottom panel was created based on homology with 1,4-β -xylanase. Shown (left) are the 8 (yellow) and 50 kDa (gray) subunits and glutamic acid residues 225 and 343 that comprise the enzyme active site (red). The heparin binding domains (HBD 1 and 2, blue and green) are in close proximity to the enzyme active site (red).

2. Heparanase as a marker of poor prognosis

Heparanase is expressed in various cancer cell lines derived from primary tumors as well as from metastatic sites by numerous methods, including RT-PCR, western blot analysis, in situ hybridization, immunohistochemistry and enzymatic activity [440-449]. Heparanase expression was often found in the invading edge of the tumor nodules by immunohistochemistry. Several studies showed that heparanase expression level may correlate with advanced tumor degree, stage, progressive tumor angiogenesis, and poor patient survival [440, 444, 450-453]. Heparanase expression correlates with poor survival in metastatic ovarian carcinoma [454]. A study in 68 breast cancer patients revealed that heparanase expression is significantly higher in

large tumors (1-5cm) than small tumors (≤ 1 cm) and the higher heparanase activity correlated with more lymphatic metastatic potential [451]. Akahashi, Hidenori, et al. reported that heparanase activity could be an independent factor to influence disease-free survival [455]. In another study, heparanase expression was found much higher than MMP2 and MMP9 in the tissue samples of 40 bladder cancer patients [456]. Study also revealed the neovascularization co-related with elevated heparanase level in the tumor tissues. Survival rate of heparanase-positive patients was much longer than heparanase-positive patients (median survival >24 months vs. 12 month) [456].

Similarly report was also observed by Rohloff, J., et *al*. who found shorter survival period (17 months) of heparanase positive pancreatic cancer patients after surgical resection of the primary tumor, compared to the heparanase negative (34 months) [440]. Increased serum heparanase activity was also reported higher and associated with a shorter survival in patients with pancreatic carcinoma patients [446]. Taken together, these observations propose that heparanase expression level in tumor tissues and/or serum might be a useful prognostic tool for postoperative survival, and as bio-markers for observing therapeutic response in malignancy.

3. Heparanase enzymatic effect in cancer

The first studies demonstrating an enzymatic activity attributable to HPSE date back to 1983, when Nakajima et al. [457] found a relationship between HS degradation and the invasive and metastatic potential of B16 melanom cells, and Vlodavsky et al. [458] demonstrated the same link in lymphomas. After the HPSE gene had been identified and cloned in the late 1990s, several experimental studies showed that this enzyme's overexpression was instrumental in increasing the dissemination and metastatic potential of cancer cells, partly as a result of the establishment of a new vascular network [443, 447]. Various gene silencing strategies for HPSE also coincided with a reduction in the invasive and metastatic properties of tumor cells [459, 460]. Enzymatic activity is optimal at low pH (5.8-6.2) that occurs at sites of ischemia such as in the center of a rapidly growing tumor. The molecular mechanism through which heparanase facilitates angiogenic responses has traditionally been attributed primarily to the release of HS-bound growth factors, such as vascular endothelial growth factor (VEGF)-A and basic fibroblast growth factor (FGF-2) [461, 462], a direct consequence of heparanase enzymatic activity.

A critical early event in the angiogenic process is degradation of the subendothelial basement membrane (BM), followed by endothelial cell (EC) migration toward the angiogenic stimulus. Similar to its involvement in tumor cell dissemination, it is conceivable that by degrading HS in the BM, heparanase may directly facilitate EC invasion and sprouting. Indeed, heparanase expression by FGF-2 stimulated, bone marrow-derived EC was demonstrated by RT-PCR [463]. Immunohistochemistry of tumor specimens revealed heparanase staining of EC in capillaries, but not mature blood vessels [463, 464]. Moreover, by releasing HS-bound angiogenic growth factors (i.e., FGF-2, VEGF) from the ECM [461], heparanase may indirectly facilitate EC migration and proliferation [463-465]. Heparanase up-regulation in such patients was associated with elevated micro-vessel density and syndecan-1 expression [466]. While heparanase is known to be proangiogenic in myeloma, which is a common feature shared with solid tumors, heparanase regulation of syndecan-1 shedding has emerged as highly relevant to multiple myeloma progression. Syndecan-1 is particularly abundant in this clinical entity, and is the dominant and often the only HS proteoglycans present on the surface of myeloma cells [444]. Cell surface syndecan-1 promotes adhesion of myeloma cells and inhibits cell invasion in vitro [467]. In contrast, high levels of shed syndecan-1 are found in the serum of some myeloma patients and are associated with poor prognosis [468]. Shed syndecan-1 becomes trapped within the bone marrow ECM where it likely acts to enhance the growth, angiogenesis and metastasis of myeloma cells within the bone [467, 469, 470]. This is supported by the finding that enhanced expression of soluble syndecan-1 by myeloma cells promotes tumor growth and metastasis in a mouse model [467, 470]. Notably, heparanase up-regulates both the expression and shedding of syndecan-1 from the surface of myeloma cells [471, 472]. In agreement with this notion, heparanase gene silencing was associated with decreased levels of shed syndecan-1 [471]. Importantly, both syndecan-1 up-regulation and shedding require heparanase enzymatic activity, because over-expression of mutated inactive heparanase failed to stimulate syndecan-1 expression and shedding [472].

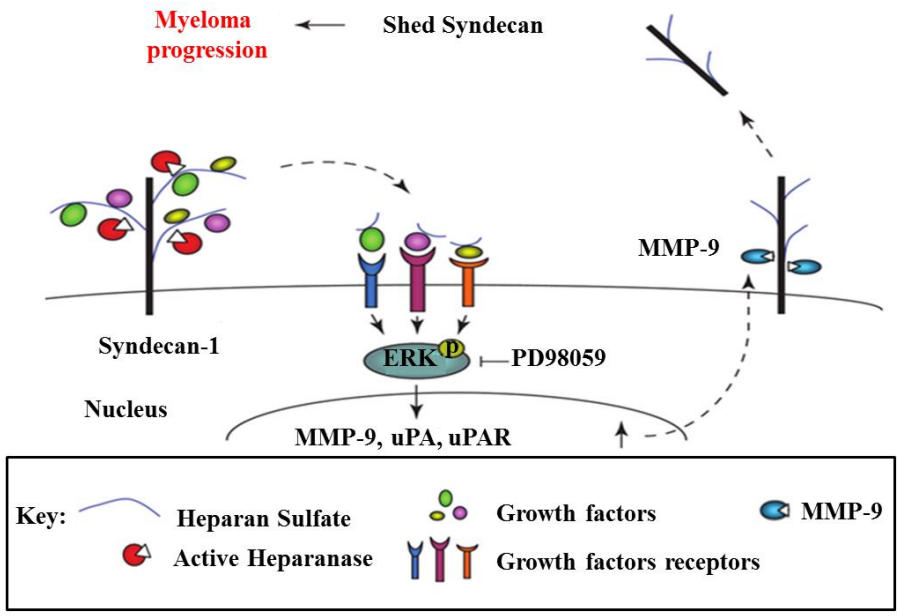

Figure 16: Remodeling of syndecan-1 by heparanase enzymatic activity modulates cell behavior and alters the tumor microenvironment
Active heparanase, secreted by tumor cells, cleaves HS side-chains of syndecan-1 and releases sequestered growth factors while still associated with small HS fragments. The HS-growth factor (i.e., HGF) complex can now interact with its high affinity receptor (i.e., c-Met) in an autocrine or paracrine manner, augmenting ERK phosphorylation. The resulting signaling induces MMP-9 expression and secretion. Similarly, the expression of uPA and uPAR is increased, resulting in MMP-9 activation. Active MMP-9 cleaves the syndecan-1 core protein, and syndecan-1 is shed from the cell surface, concentrates in the tumor microenvironment and facilitates myeloma progression.

The clinical significance of the enzyme for tumor progression emerges from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, in situ hybridization, RT-PCR and real time-PCR analyses revealed that heparanase is up regulated in the majority of human tumors examined. These include carcinomas of the colon [1,2], thyroid [473], liver [474], pancreas [440, 475], bladder [456, 476], cervix [477], breast [451], gastric [478, 479], prostate [480], head and neck [448, 481], as well as multiple myeloma [466], leukemia and lymphoma [482]. In most cases, elevated levels of heparanase were detected in about 50% of the tumor specimens, with a higher incidence in pancreatic (78%) and gastric (80%) carcinomas, as well as in multiple myeloma (86%). In all cases, the normal looking tissue adjacent to the malignant lesion expressed little or no detectable levels of heparanase, suggesting that epithelial cells do not normally express the enzyme. In

several carcinomas, most intense heparanase staining was localized in the invasive front of the tumor [448, 476, 479], supporting a role of heparanase in cell invasion. Furthermore, patients that were diagnosed as heparanase-positive exhibited a significantly higher rate of local and distant metastasis as well as reduced post-operative survival, compared with patients that were diagnosed as heparanase-negative [440, 466, 476, 479, 483]. In addition, heparanase up regulation in primary human tumors correlated in some cases with tumors of larger size [451, 474, 479], and with enhanced micro vessel density [456, 466, 474, 483], providing a clinical support for the pro-angiogenic function of the enzyme. Collectively, these studies provide a strong clinical evidence for the pro-metastatic and proangiogenic functions of heparanase.

4. Heparanase non-enzymatic effect in cancer

Experiments applying heparanase lacking enzymatic activity due to point mutations (Glu225, Glu343) in its active site, have demonstrated that this endoglycosidase also exerts non-enzymatic activities, independent of its involvement in ECM degradation. Enzymatically inactive heparanase was noted to facilitate adhesion and migration of primary endothelial cells [484] and to promote phosphorylation of signaling molecules such as Akt and Src [484-486], the latter found responsible for VEGF-A induction following exogenous addition of heparanase or its over-expression [484, 486]. Furthermore, heparanase was also reported to facilitate the formation of lymphatic vessels. In head and neck carcinoma, high levels of heparanase were associated with an increase in lymphatic vessel density, tumor cell invasion to lymphatic vessels, and expression of VEGF-C [487], the latter being a potent mediator of lymphatic vessel formation [488]. Activation of Epidermal growth factor receptor (EGFR) appears to be markedly elevated in cells over-expressing heparanase or following its exogenous addition, while heparanase gene silencing is accompanied by reduced EGFR and Src phosphorylation levels [489]. Notably, EGFR activation was observed following the addition or over-expression of mutated, enzymatically inactive heparanase protein.

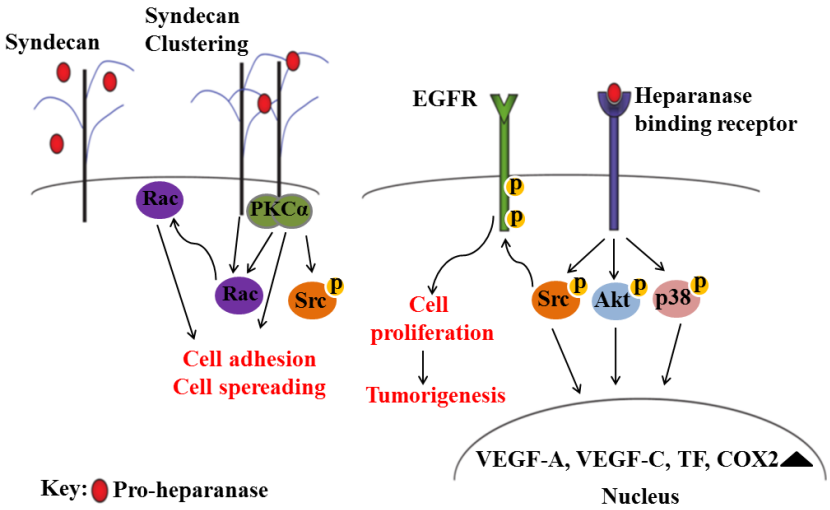

Figure 17: Enzymatic activity-independent function of heparanase at the cell surface. Enhanced cell adhesion and spreading is mediated by the recruitment and activation of PKCα, Rac1, and Src. In addition, heparanase is thought to interact with a heparanase binding cell surface protein/receptor, leading to HS-independent Akt, p38, and Src activation. This results in enhanced transcription of genes such as vascular endothelial growth factor (VEGF-A, VEGF-C), tissue factor (TF), and Cox2, and further contributes to cell adhesion, spreading and motility.

5. Heparanase non-enzymatic pro-coagulant activity

Immunohistochemical studies revealed that many tumors express high levels of tissue factor (TF) raising the possibility of TF role in the pathogenesis of cancer [490, 491]. Heparanase over-expression in cancer cells resulted in a marked increase in TF levels as assessed by immunoblot and real-time PCR analyses [492]. Likewise, TF was induced by exogenous addition of recombinant heparanase to tumor cells and primary endothelial cells. This induction was mediated by p38 phosphorylation and correlated with enhanced pro-coagulant activity. TF induction was further confirmed by heparanase over expression in transgenic mice and, moreover, correlated with heparanase expression levels in leukemia patients [492]. Recently, it was demonstrated that heparanase may serve as a cofactor of TF, suggesting its direct involvement in activation of the coagulation cascade [493]. These findings were supported by experiments indicating that heparanase increases the level of factor Xa in the presence of TF/VIIa and the effect is enzymatically independent. The newly generated Xa had the same molecular weight as Xa cleaved by TF/VIIa and was active as depicted by increased conversion of prothrombin to thrombin. The finding of

increased Xa generation in the presence of heparanase was confirmed in the clinical setting, using a newly developed assay for the evaluation of heparanase procoagulant activity [494-496]. Thus, apart from the ability of heparanase to increase Xa levels in normal human plasma, a statistically significant positive correlation between the plasma levels of heparanase and Xa was found in patients with acute leukemia and healthy donors [493]. Tissue factor pathway inhibitor (TFPI) is a plasma Kunitz-type serine protease inhibitor and the only known endogenous modulator of blood coagulation initiated by TF [497, 498]. Exogenous addition or over expression of heparanase in transfected cells led to release of TFPI from the cell surface and its accumulation in the cell culture medium [499]. Importantly, the in vitro studies were supported by elevation of TFPI levels in the plasma of transgenic mice overexpressing heparanase. Moreover, increased levels of TFPI have been noted in the plasma of cancer patients [500, 501], reflecting, possibly, induction of heparanase expression and elevation of its plasma levels, as revealed by ELISA assay [502]. In human umbilical vein endothelial cell (HUVEC) and tumor derived cell lines, release of TFPI from the cell surface correlated with enhanced TF-mediated coagulation. This effect was evident as early as 30 min following heparanase addition, and prior to the induction of TF [492] or TFPI expression. Thus, heparanase enhances local coagulation activity through two independent mechanisms: induction of TF expression [492], and TFPI dissociation from the cell surface. Both functions require secretion of heparanase, but not its enzymatic activity. The underlying mechanism is apparently release of TFPI due to its physical interaction with the secreted heparanase, as is clearly evident from co-immunoprecipitation experiments [499], reflecting a functional interaction between heparanase and a membrane protein.

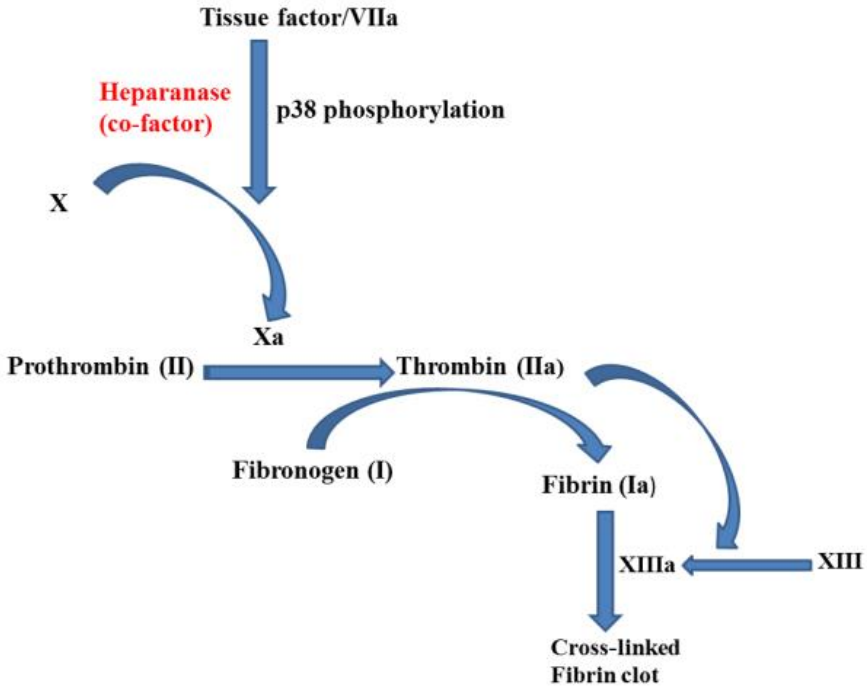

Figure 18: A model of heparanase procoagulant domain effects.
Heparanase directly enhances tissue factor (TF) activity which leads to increased factor Xa production and subsequent activation of the coagulation system.

6. Heparanase and Fibrosis

Tissue fibrosis is an unregulated wound-healing response characterized by a gradual accumulation and decreased remodeling of ECM. Chronic injuries are the main triggers of this process in major organs [503]. Common pathways and effector cells are implicated in fibrosis in various parenchymal organs. Persistent parenchymal cell injury leads to chronic inflammation that, in turn, stimulates the activation of effector cells into fibrogenic myofibroblasts. Myofibroblasts express the alpha isoform of smooth muscle actin (α-SMA) and secrete large amounts of ECM proteins (primarily collagen, fibronectin and laminin), which are responsible for tissue scarring and organ architecture deformation and failure. The source of the myofibroblast pool varies, including resident fibroblasts, fibrocytes, pericytes and epithelial cells undergoing epithelial-to-mesenchymal transition (EMT) [504]. The trans-differentiation of epithelial cells into myofibroblast-like cells (i.e. EMT) is characterized by the loss of epithelial markers and the acquisition of mesenchymal ones, and by an increase migration and matrix protein secretion [189]. Increasing interest has been paid to HPSE in this field in the last decade, since its involvement in the EMT of renal tubular cells in kidney fibrosis was discovered. HPSE is overexpressed by injured

glomerular and tubular cells exposed to albuminuria, high glucose levels and advance glycosylation end products [505]. Once secreted, HPSE is a key regulator of FGF-2 and TGF-β activity, the main pro-fibrotic factors and inducers of EMT in the kidney [506, 507]. HPSE knock-down in a proximal tubular cell line prevents the EMT induced by FGF-2 [506], and delays the EMT induced by TGF-β [507]. A lack of HPSE also prevents TGF-β overexpression by injured tubular cells in vitro [507], and HPSE-ko mice show no increase in TGF-β in injured kidneys, and no onset of fibrosis [508]. These findings point to HPSE as a potential pharmacological target in chronic renal disease [509]. There are few and sometimes conflicting data on the role of (HPSE) in promoting fibrosis in organs other than the kidney. HPSE regulates TGF-β availability by shedding syndecan-1, and its upregulation has consequently been found associated with intestinal fibrosis in vivo [510]. Unlike the well-known role of HPSE as a pro-metastatic agent in hepatocellular carcinoma, the involvement of HPSE in the pre-cancerous condition of liver fibrosis/cirrhosis is still not clear. Ikeguchi et al. found an inverse correlation between HPSE expression and stage of liver fibrosis in human tissue [511]. Xiao et al. reported no difference in HPSE expression between cirrhotic and normal liver tissue [512]. Discordant data have also emerged from a rat model of chronic liver disease: Ohayon et al. found an increase of HPSE in advanced fibrosis [513], whereas Goldshmidt et al. had previously described HPSE protein levels peaking in the early stages of fibrosis [514].

The activity of heparanase closely correlates with tumor invasion, metastasis [515, 516] and fibrosis [507] and the enzyme is moderately up-regulated, especially in metastatic cancers. Based on previous reports, we postulated that heparanase would be involved at invasion, metastasis and fibrosis of gastric cancers cells. In this regard, Friedmann et al. identified the expression of heparanase in dysplastic and neoplastic colonic mucosa and the evidence for its role in colonic carcinogenesis [443].

7. Heparanase inhibitors

Heparanase enzymatic activity is important in the advancement of tumor angiogenesis, primary tumor growth, invasion, and metastasis. Heparin is the best natural choice for heparanase inhibitor but cannot be used as anticancer drug due to its potent anticoagulant activity [517]. Extensive struggle has been done for the improvement of modified heparins and related polysulfated compounds with less

anticoagulant activity. Current advancement in drug development directed to numerous classes of heparanase inhibitors, containing chemically modified natural products, small molecule inhibitors, and antibodies. Some heparanase inhibitors validated as potent active to inhibit tumor angiogenesis, tumor progress, or tumor metastasis are listed below

Drug name	Drug category	Mechanism of action	Clinical trial	Note	Ref.
PI-88 (Mupafostat)	Heparan sulfate mimetic	Inhibits heparanase; ↓FGF-1, FGF-2, and VEGF	Phase III in HCC (NCT01402908)	Also releases TFPI; ↓angiogenesis	[518-521]
PG545	Heparan sulfate mimetic	Inhibits heparanase; ↓FGF-1, FGF-2, and VEGF	Phase I in advanced solid tumors (NCT02042781)	In lymphoma exerts major antitumor effects by ↑NK cell activity	[522, 523]
SST0001 (roneparstat)	Heparan sulfate mimetic	Inhibits heparanase; ↓HGF, VEGF, and MMP-9. ↓shedding of syndecan-1	Phase I in R/R multiple myeloma (NCT01764880)	↓Regrowth of myeloma tumors in vivo after completion of chemotherapy	[524, 525]
M402 (necuparanib)	Heparan sulfate mimetic	Inhibits heparanase; ↓ EC sprouting, FGF2, HB-EGF, and VEGF	Phase I/II trial for the treatment of metastatic pancreatic cancer (NCT01621243)		[526]
Suramin	Small molecule inhibitor	Inhibits heparanase; ↓FGF-2 and caspase-3	None	↑HSPGs, ↓fibrosis and hepatic tissue breakdown in HCC murine model	[527, 528]
OGT2115	Small molecule inhibitor	Inhibits heparanase	None	Suppresses metastasis induced by endoplasmic reticulum stress from chemotherapy in breast cancer cells	[529]
9E8 and H1023	Antiheparin antibodies	Inhibition of heparanase; ↓cellular invasion and metastasis	None	Potent synergism when combination of both antibodies is used in myeloma and lymphoma murine models	[530]
B-Cell MAP	Vaccine	Inhibits heparanase; ↓expression of	None	↓Microvessel density and tumor volume in HCC murine	[531]

		VEGF and FGF2		model	
T-Cell MAP	Vaccine	CTL dependent lysis of tumor cells, ↑IFN-γ	None	No activity against autologous lymphocytes and dendritic cells	[532]
Defibrotide	Nucleic acid-based inhibitors	↓Heparanase expression and tumor growth	Phase I/II trial in R/R myeloma with combination chemotherapy (NCT00406978)	No direct cytotoxic effect on myeloma	[533, 534]
Single-strand DNA aptamers	Nucleic acid-based inhibitors	↓Expression of heparanase; ↓tissue invasion of tumor cells	None	No direct cytotoxic effects on oral cancer cells; stable, with ↓complex formation	[535]
RNAi (siRNA/shRNA)	Nucleic acid-based inhibitors	↓Expression of heparanase; ↓invasion and migration of tumor cells	None	Found to work in multiple cell lines including HCC and melanoma	[536-538]

Table 5: Heparanase inhibitors currently in development

8. Suramin

Suramin, a polysulfonated naphthyl urea, was first used against African parasitic infections, such as Rhodesian and Gambian trypanosomiasis. Later, it was reported that suramin also inhibited heparanase with an IC50 of 48 mM. Suramin inhibited cell growth and exerted antiangiogenic and anti-metastatic effects through blocking the activity of heparanase [539].

Due to its anti-proliferative activity against numerous human cancer cell lines in dose- and time-dependent manner [540], suramin alone or combined with cytotoxic drugs has been studies in many clinical trials that include ovarian cancer [541, 542]. Suramin inhibitory activity against heparanase could be improved by increasing the size of R group from methyl to ethyl, or tert-butyl group in its chemical structure [539]. New suramin analogues have now been established to improve this antitumor activity and overcome its side-effects [543].

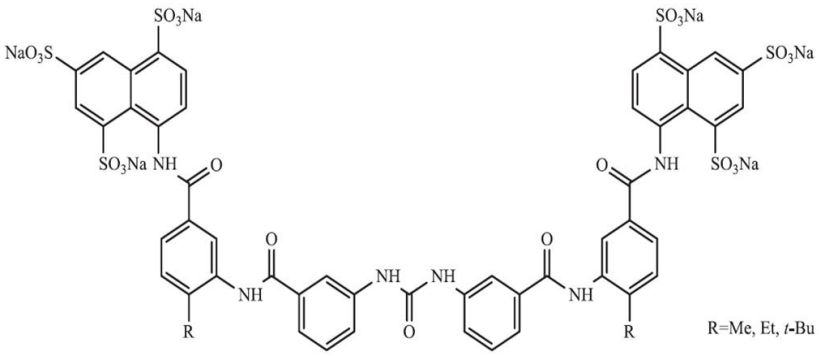

Figure 19: Molecular structure of Suramin.
Suramin (8,8′-carbonyl-bis [imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimino] bis- 1,3,5-naphthalene-trisulfonic acid)

Chapter 7: Coagulation and Cancer

1. Coagulation biology

Hemostasis is a complex system that is based on various physiological phenomena which aim is to maintain the blood flow within the vessels. These mechanisms contribute to stop the bleeding and to prevent thrombosis. Hemostasis is based on the action of various cellular players such as the endothelium, the vessel wall, platelets, circulating blood cells and various plasma proteins. Classically, coagulation can be distinguished into three main phases that are activated simultaneously: primary hemostasis, coagulation and fibrinolysis. These three phases are concurrent and interdependent, even if they differ in duration and intensity. The importance of each of these mechanisms varies, also depending on the location of vascular lesion (arterial vessel or venous) and the size of the vessel.

1.1 Primary hemostasis

Primary hemostasis is activated immediately after the formation of a vascular gap. Its role is to close the wound and stop bleeding by forming a platelet clot, known as "white thrombus". At the onset of a vascular lesion, primary hemostasis process takes place. The first reaction triggered is the localized vasoconstriction and platelets activation. This reaction stops bleeding, reduces blood flow and changes hemodynamic conditions. The exposure of the sub-endothelium to the von Willebrand factor (*von Willebrand factor:* vWF) induces the recruitment of other platelets. Platelets aggregate and form a fragile microthrombus [544]. This induced microthrombus and degranulation of platelets result in the release of cytokines (VEGF, PDGF, IGF-1, TGF-β) and coagulation factors (V, vWF and fibrinogen) [545]. The aggregation is irreversible and the clot solidifies, thus forming a platelet plug.

1.2 Coagulation

Coagulation is the result of a chain of chemical reactions that involve various substrates and enzymes, leading to the formation of a fibrin clot. The key enzyme for converting fibrinogen, a soluble protein into soluble fibrin is thrombin. The process of formation of thrombin is complex and involves various clotting factors (I to XIII).

Clotting factors are predominantly synthesized in liver as protein zymogens. They are presented in Table. The coagulation process is based on two pathways:

1.2.1 The extrinsic pathway:

Tissue factor (TF) is a membrane receptor, embedded in the vessel wall and exposed to blood following vascular injury. The TF activate factor VII to form a complex which, at a high concentration, activates factor X. In the presence of phospholipids membrane and calcium, factor Xa binds factor Va in turn to form the prothrombinase enzyme required for the conversion of prothrombin (II) to thrombin (IIa). The generated thrombin (IIa) triggers a positive feedback by activating the platelets and factors V, VIII and XI. Thrombin enhances platelet aggregation within the clot by binding to the surface of the platelets to its PAR-1 receptor (protease-activated receptor 1) [546]. The extrinsic pathway is quick and comes early to generate the first molecules of thrombin [547].

1.2.2 The intrinsic pathway:

It is triggered by the activation of factor XII after contact with collagen. The activation of factor XII leads to the conversion of prekallikrein (PK) to kallikrein (PKa). A self-activation loop between the PKa and factor XII, is set up. Finally, cascade of reactions allow the formation of prothrombinase (factor Xa and Va) and ultimate thrombin [548].

Thrombin generated by these two channels, cleaves fibrinogen and releases fibrin monomers. Factor XIIIa stabilizes fibrin through covalent bonds, thereby forming a solid network around the platelet aggregates. The coagulation process ends when the gap is closed by platelet deposition and fibrin.

Common name	Symbol	Other names	Site of synthesis	Function	Final product
Fibrinogen	I	Factor I	Liver	Fibrin precursor	Fibrin
*Prothrombin	II	Factor II	Liver	Protease zymogen (plasma)	Thrombin IIa
Tissue factor	TF	Thromboplastin factor, Factor III	Subendothelium blood cells	Initiator, co-factor VIIa in IX and X activation	Immediately active
Calcium	IV	Ca2+	ubiquitous	Cofactor	
Factor V	V	Proaccelerin	Liver	Xa cofactor in the activation of prothrombin (plasma, platelets)	Va
Factor VI: not retained by the International Nomenclature Committee					
Factor VII *	VII	Proconvertin	Liver	Protease zymogen (plasma)	VIIa
Factor VIII	VIII	Antihemophilic factor A	Liver	Cofactor IXa in the X activation (plasma)	VIIIa bound to vWF
Factor IX *	IX	Antihemophilic factor B	Liver	Protease zymogen (plasma)	IXa
Factor X *	X	Stuart factor	Liver	Protease zymogen (plasma)	Xa
Factor XI	XI	Plasma thromboplastin	Liver	Protease zymogen (plasma)	XIa

		antecedent			
Factor XII	XII	Hageman factor	Liver	Protease zymogen (plasma)	XIIa
Factor XIII	XIII	Fibrin stabilizing factor	Liver	Transgluta minase zymogen (plasma, platelets)	XIIIa

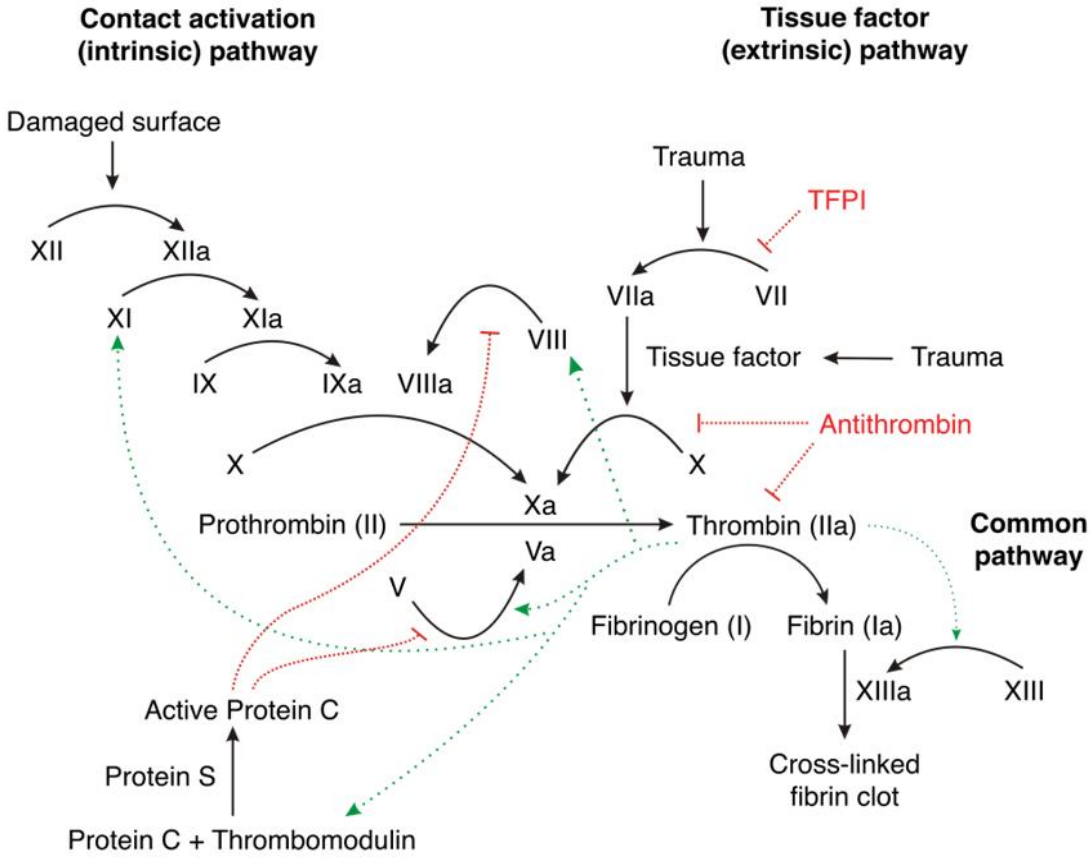

Figure 20: Summary diagram of the regulation of coagulation.

1.3 Fibrinolysis

When healing occurs to repair the vascular injury, the fibrin clot gradually dissolves. Hydrolysis of fibrin is ensured by plasmin, a serine protease secreted by hepatic zymogen: plasminogen. Plasminogen is activated by two proteases: the first is of renal origin, named urokinase (uPA) and the second is of endothelial origin, called tissue-plasminogen activator (t-PA). Actually, the presence of fibrin leads to activation of the prourokinase to urokinase. The activation of t-PA is done by plasmin substrate, which engages a strong loop auto-activation [549]. Plasmin generated degrades the fibrin and releases very heterogeneous fragments called fibrin degradation products (FDP) as D-dimer.

Fibrinolysis is controlled by different routes:

- Inhibition of plasmin: when plasmin is in excess, it is inactivated in plasma by its inhibitors, such as α2-antiplasmin and a lesser degree α2- macroglobulin [550].
- Internalization of u-PA / u-PAR complex by cadherin dependent endocytosis: This way is activated following the binding of the complex with PAI-1 (plasminogen *activator inhibitor*- 1). This results in degradation of u-PA and recycling of its receptor. PAI-2, a product of the alternative splicing of PAI-1, acts as an inhibitor of both u-PA and t-PA.
- Inhibition by TAFI (Thrombin-activatable fibrinolysis inhibitor): This is a carboxypeptidase. Its activity is improved in the presence of thrombomodulin (TM), a peptidoglycan expressed on the surface of endothelia [551].

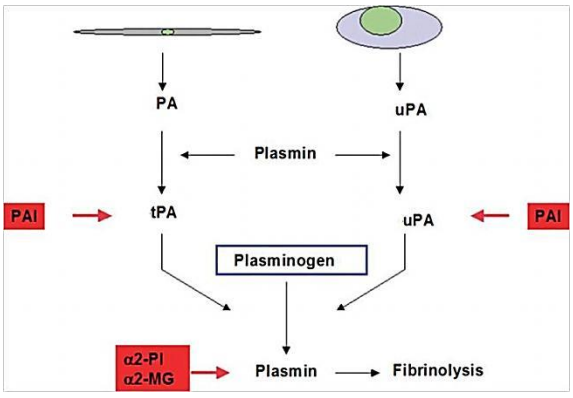

Figure 21: Molecular mechanisms of fibrinolysis.
Plasminogen is converted to activate serine protease, plasmin, through the action of tPA and uPA. Both activators are secreted by endothelial cells and macrophages monocytes or endothelial cells respectively. The red blocks represent the negative regulatory pathways.

2 Inhibition of fibrin

Inhibition of fibrin formation is one of the major mechanisms of negative regulation of coagulation. It is based on three routes:

- The *tissue factor pathway inhibitor* (TFPI): TFPI forms a complex with factor Xa, TF and factor VIIa, thus blocking the extrinsic coagulation pathway [552].
- Antithrombin (AT): This is a liver protein of the serpin family. It is capable of forming a complex with thrombin and factor VIIa, IXa, Xa, XIa and XIIa and neutralize irreversibly [553].
- The protein C / endothelial protein C receptor: Protein C (PC) and its endothelial receptor (EPCR: *Endothelial protein C receptor)* are central to anticoagulant systems. Indeed, when interacting with thrombomodulin, the thrombin activates a feedback inhibition process and loses its coagulant properties following conformational changes. Thrombin even loses its affinity for platelets and fibrinogen and becomes a major player in the inhibition of fibrin [554]. The thrombin-TM is responsible for the activation protein C, hepatic vitamin K-dependent serine protease. Activated protein C (aPC) is thus released. In the presence of protein S, aPC inactivates factor Va and factor VIIIa by proteolytic cleavage, thereby inhibiting the generation of thrombin.

In addition to its anticoagulant activity, the PC is also able to improve fibrinolysis by inactivating PAI-1 secreted by endothelial cells [555]**.**

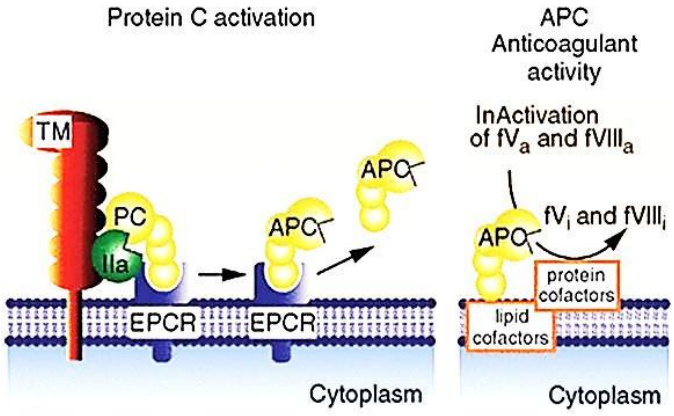

Figure 22: Role of protein C and its receptor in the inhibition of fibrin.

3 Coagulation and carcinomatosis

Malignancy disturbs the hemostatic system and the hemostatic system affects malignancy. As solid tumors rely upon vascular network for their nourishment, it follows that interference with that supply may compromise tumor growth. Several studies have proposed that the main difference between normal and tumor-related blood vessels is their tendency of blood coagulation within tumor vessels. The association between tumors and abnormalities of the blood coagulation system was first recognized as long ago as 1865, when French clinician Armand Trousseau reported the frequent occurrence of venous thrombosis in patients with gastric carcinoma [556]. Thrombosis is the most frequent complication and the second cause of death in patients with overt malignant disease [557].

More than 50 years ago, Levin and Conley reported that thrombocytosis was associated with breast, lung, digestive and ovarian cancers [558]. Hemostatic disorders in cancer result from the capacity of tumor cells to secrete procoagulant factors and to interact with blood components such as platelets [559, 560]. While platelets are essential for normal hemostasis, their unbridled activation may result in thrombus formation leading to thrombocytosis complications. Moreover, patients with elevated platelet count have a higher risk to develop venous thromboembolism [561].

The coagulation/fibrinolytic system is responsible for the intravascular fibrin homeostasis besides participating in a wide variety of physio-pathological processes such as tumor growth, invasion and metastasis [562, 563].

4 Role of factor XII zymogen, tissue factor and fibrin in angiogenesis

The formation of new cellular masses at an organ site creates an obvious extra demand for oxygen, growth factors, and metabolites and increases the need for tumor-associated blood vessels. Although most investigations focus on FXII as a serine protease, zymogen FXII has mitogenic activities on cultured cells independent of its enzymatic activity. Evidence indicated that FXII stimulates aortic sprouts from wild-type mice but not from uPAR−/− aorta and initiates new vessel formation into matrigel plugs in wild-type but not in uPAR-deficient animals. Vice versa, there is less number of vessels in skin punch biopsies in a FXII−/− mouse model both constitutively and in a wound healing model [564]. In contrast, in another FXII−/− mouse strain, there are no obvious vascular abnormalities in histologic analyses [565].

These combined data indicate that zymogen FXII, like single-chain urokinase, functions as a growth factor that mediates cell signaling leading to proliferation and stimulating angiogenesis, indicating a new in vivo activity for zymogen XII in postnatal angiogenesis after ischemia, inflammation, and injury.

Recent data suggested that increased expression of TF in tumors may contribute to angiogenesis in part by increasing VEGF protein expression and downregulating the expression of the antiangiogenic protein thrombospondin [566]. For example, TF-positive colorectal tumors have higher levels of microvessel density and VEGF expression than TF-negative colorectal tumors do [567].

In the coagulation dependent pathway, the interaction of TF and factor VIIa induces Ca2+ oscillations and changes in gene expression, and the formation of the TF-factor VIIa complex leads to the activation of the mitogen-activated protein kinase (MAPK) pathway [568], which is a major inducer of VEGF expression. Colocalization of upregulated PAR-2 with phosphorylated TF occurs only in neovessels. The phosphorylation of TF appears to be the mechanism that switches off the negative regulatory control that promotes pathologic PAR-2-dependent angiogenesis [569].

Fibrin-induced neovasculation is based on clotting related mechanisms that involve platelet activation and fibrin deposition. Cross-linked fibrin has been found in different human malignant tumors. It is present in the endothelium of angiogenic vessels of invasive cancer specimens but not in vessels of benign tumors [570]. Fibrin can bind to inflammatory cells or to tumor cells and is deposited around tumor cells as scaffolding that promotes further angiogenesis. Fibrinogen and fibrin fragments, such as fragment E, have been shown to stimulate angiogenesis both in vitro and in vivo [571]. The binding of endothelial cells (ECs) to fibrin with the involvement of the adhesion molecule vascular endothelial cadherin may be necessary for capillary tube formation, a critical step in angiogenesis. The fibrin matrix that develops around tumors provides a provisional proangiogenic environment that supports vessel formation and stimulates EC proliferation and migration [572]. The fibrin matrix can promote a proangiogenic response by upregulating the expression of αvβ3 integrin receptor to facilitate EC migration and capillary formation [573].

5 Cell adhesion to fibrin

The association between coagulation factors and malignancy was recognized more than a century ago [574]. Positive fibrin staining is frequently found in focal sites at the interface of the tumor cells and surrounding stroma, but not in the ECM of normal host cells. Much like in a healing wound, the deposition of fibrin/fibrinogen, along with other adhesive glycoproteins, into the extracellular matrix (ECM) serves as a scaffold to support binding of growth factors and to promote the cellular responses of adhesion, proliferation, and migration during angiogenesis and tumor cell growth [575]. Fibrin surrounding tumors may protect them from infiltrating inflammatory cells by acting as a barrier, thus preventing inflammatory reactions directed towards the tumor cells [576, 577].

In carcinomatosis, the presence of cancer cell in peritoneal cavity induces the pro inflammatory state and modified the mesothelial cell layer microenvironment [578]. The mesothelial layer disrupted and loses their polarity [579]. The contact between mesothelial layers is broken and mesothelial cells are exfoliated from the surface leaving empty space. A quantitative change in synthesis and degradation of ECM proteins occurs. This imbalance between these two processes may result in tumor stroma containing increased amounts of collagen, FN, proteoglycans [580], and, perhaps, fibrinogen [575]. Thus, tumor stroma may be considered “wound healing gone awry” and tumors themselves may be considered “wounds that do not heal” [581]. The source of some of these matrix proteins is thought to be from leaky blood vessels [582] or inappropriate synthesis by the tumor cells. Some consequences of inappropriate or modified synthesis of ECM molecules in the context of tumorigenesis include changes in proliferation, induction of angiogenesis, altered cell adhesion and cell migration, and ultimately tumor metastasis. Cancer implies aberrant cell growth. A combination of autocrine and paracrine growth factors and cytokines and ECM molecules leads to degregulation of cell cycle control, which results in increased cell proliferation and malignant transformation [580].

6 Soluble Endothelial protein C receptor

EPCR is a type 1 transmembrane glycoprotein that shares considerable homology with the major histocompatibility complex [15]. Endothelial protein C is an important regulator of homeostasis in addition to its involvement in the systemic response to

acute inflammation. EPCR gene carries 13 single nucleotide polymorphisms, which define 3 haplotypes, 1 of which (A3), that lacks the transmembrane and cytoplasmic domains was strongly associated with high sEPCR levels. As A3 haplotype is responsible for a high sEPCR level, it is a candidate risk factor for venous thrombosis [583, 584].

EPCR exists as membrane bound as well as free soluble form (sEPCR). EPCR is known to be constitutively released in the plasma in a free soluble form as a result of its proteolytic cleavage. Soluble EPCR (sEPCR) has the ability to trap free aPC, thereby depriving it of its anti-coagulant function within the surrounding environment [585, 586]. The shedding of EPCR is known to be regulated by IL-1β (interleukin), TNF-α, endotoxin, and via the MAP kinase signaling pathways in human vascular endothelial cell line (HUVEC) [587] and by the presence of EPCR A3-haplotype homozygosis [588]. sEPCR, infact, can regulate the quantity of circulating aPC.

Beaulieu and Church (2007) claimed that aPC increases breast cancer cell invasion and chemotaxis through EPCR and PAR-1. These findings are demonstrative of the importance of EPCR expression in tumor cells [589]. Presence of sEPCR in plasma influences innate immune response [590] and likely is a biomarker of cancer-associated hypercoagulability [591].

It is known that circulating protein C zymogens, secreted by liver, binds to endothelial protein C receptor (EPCR) with high affinity and stimulates its activation via the thrombin-thrombomodulin complex. Interest in EPCR/aPC in relation to tumor biology is gaining momentum with the recent appearance of an increasing number of publications [592-595]. The activated protein C (aPC), together with its cofactor protein S, degrades factors Va and VIIIa and thereby interferes with thrombin generation and inhibits the coagulation cascade [596-598].

The increase in plasmatic level of sEPCR in cancer patients leads to increased sequestration/capture and use of protein C. This could affect or modulate thrombotic events and immune inflammatory response.

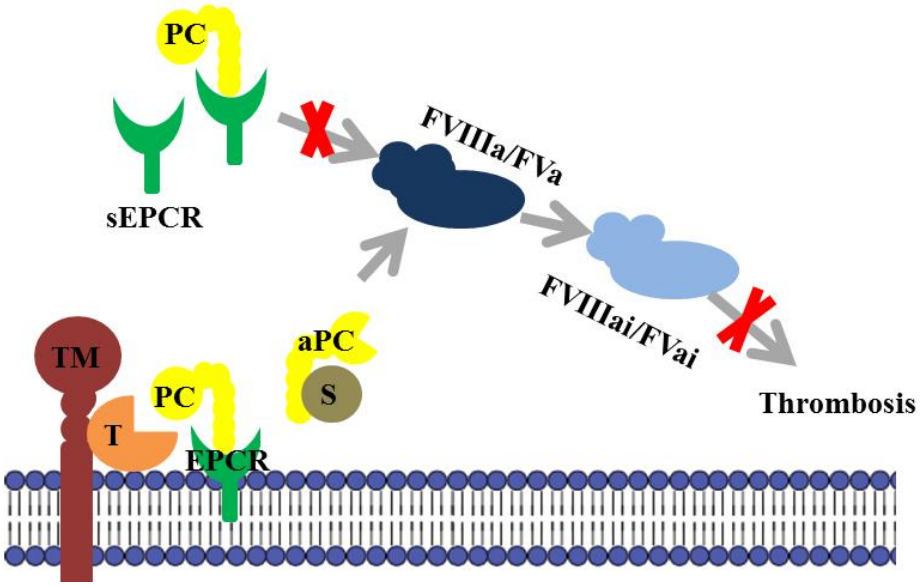

Figure 23: Schematic drawing of the anticoagulant properties of membrane associated EPCR

7 Thrombopoietin

The term thrombopoietin (TPO) first appeared in the literature in 1958 [1], to depict the primary regulator of platelet production [599]. TPO is the key cytokine involved in the regulation of thrombopoiesis. TPO is constitutively expressed, but the level of circulating TPO is directly related to platelet mass [600-602]. TPO is mainly produced by the liver and it is also secreted by kidney, bone marrow and spleen [603]. The human TPO gene is localized on chromosome 3q27 and comprises six exons and five introns [604-606]. To date, 8 different variants of TPO mRNA have been identified, including the full length mRNA (TPO-1) and its 7 alternative splicing variants. Proliferative activity was highlighted only in TPO-1 isoform [607].

Moreover, TPO seems to be more than a megakaryopoiesis regulator. The JAK-STAT signal transduction pathway is activated upon binding of TPO to its receptor, resulting in changes in gene expression that promote megakaryocytic and cancer [33, 34]. Indeed, TPO has been admitted as a crucial regulator of proliferation and secretory activity in porcine ovarian follicular cells [608]. In pathologic conditions, several cancer cell lines from lung, stomach, liver and thyroid human carcinomas express the TPO gene [609]. In a case report, Furuhashi *et al* reported that TPO could be produced by ovarian carcinoma [610]. Tsukishiro and his colleagues observed, in a

comparative study, that plasma TPO concentration may be a biomarker that distinguishes between benign tumor patients and those with malignant ovarian cancer [611]. It was already described that TPO level increased by an inflammatory process mediated by IL-6 , produced by macrophages and monocytes, dose-dependently increases TPO mRNA levels in hepatoma cell lines [612]. However, the presence of TPO detected by immunohistochemistry attributed to the capture of TPO due to TPO receptor expression in human cancer cells [613].

Although great progress has since been made in unraveling the molecular and cellular basis of this phenomenon, its significance remains something of a mystery. Data from several studies reported that the hemostatic components and the cancer biology are interconnected in multiple ways. This opens the way to the development of bifunctional therapeutic approaches that are both capable of attacking the malignant process and resolving the coagulation impairment.

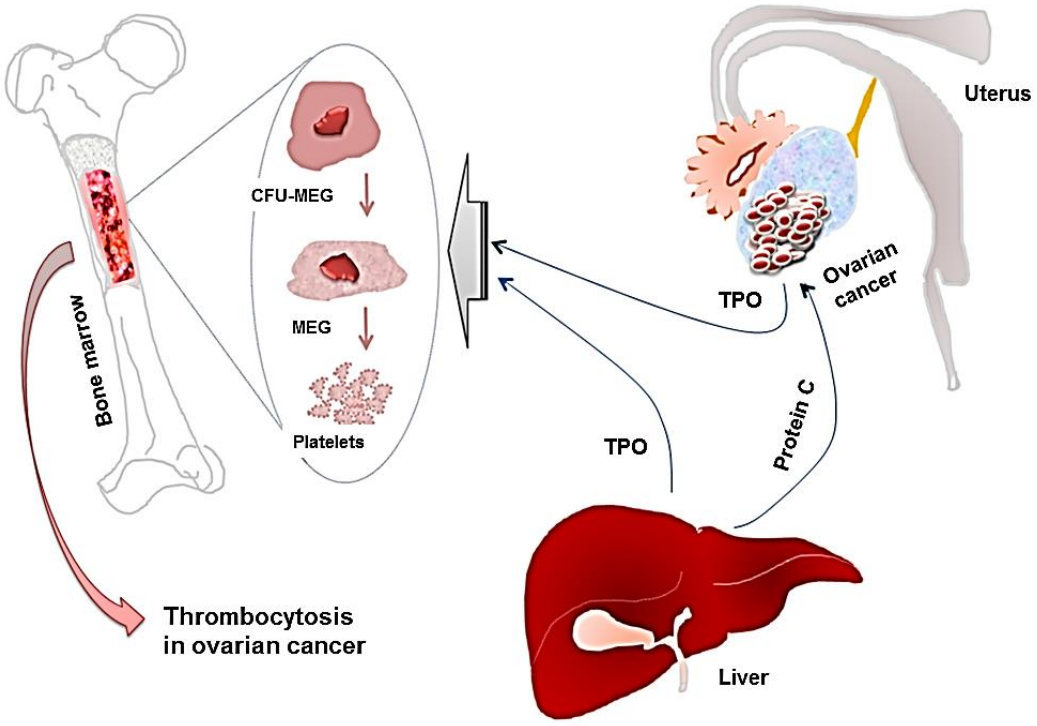

Figure 24: Platelet-ovarian cancer signaling pathways and potential therapeutic targets. aPC secreted by liver stimulates thrombopoietin (TPO) production from ovarian cancer cells, which drives thrombopoiesis in the bone marrow and thrombocytosis.

OBJECTIVES

The development of targeted therapies is the main objective of the cancer research. With the aim of better understanding and control of different components of the tumor process, these research studies inspect all the mechanisms involved in this pathology. Thus, the work presented here is from this perspective.

Gastric signet ring cell adenocarcinoma (SRCA) is unique among gastric carcinoma characterized by remarkable fibrosis, rapid invasive progression and high frequency of metastasis to the peritoneum. Abundant evidences have revealed the importance of epithelial-mesenchymal transition (EMT) in promoting metastasis in SRCA when genetically unstable cancer cells/cancer stem cells (CSCs) adapt to a tissue microenvironment that is distant from the primary tumor.

EMT is a characteristic of the majority of metastatic cells. Dissemination of tumor cells from the primary cancer may occur by peritoneal circulation, associated with ascites formation. This results in invasion by exfoliation of mesothelial cells from peritoneal surface to form cancer nodules.

Heparanase is an endo-β-D-glucuronidase enzyme, capable of cleaving heparan sulfate side chains of heparan sulfate proteoglycans on cell surfaces and the extracellular matrix, which further regulate the bioavailability of growth factors (FGF-2, TGF-β). These growth factors play a major role in tubulo-interstitial fibrosis of chronic kidney disease.

Heparanase may also affect the hemostatic system in a non-enzymatic manner. Heparanase up-regulate the expression of the blood coagulation initiator tissue factor (TF) on the cell surface membrane of tumor cells, leading to increase cell surface coagulation activity.

Moreover, heparanase is significantly correlated with the invasion and metastasis of malignant cells in vitro. Neprilysin, a cell surface zinc dependent metalloprotease and other endopeptidases, are also associated with the tumor progression of a large variety of cancers, such as breast, gastric and colorectal carcinomas.

EPCR, a negative regulator of coagulation appears to be important for protein C activation by cancer cells. Activated protein C upregulates ovarian cancer cell migration and promotes unclottability of the cancer cell microenvironment.

Interleukin-6 secreted by ovarian cancer cells stimulates hepatic thrombopoietin (TPO) production, which drives thrombopoiesis in the bone marrow. TGF-β secreted from thrombocytes interacts with cancer cells to increase proliferation. TPO could be produced by ovarian carcinoma.

Poor differentiation is an important hallmark of cancer cells, and differentiation therapy holds great promise for cancer treatment. Direct differentiation could be a breakthrough for overcoming CSCs. Suramin, an inhibitor of heparanase, alone or combined with cytotoxic drugs has also been studies in many clinical trials that include ovarian cancer. Cancer prevention and anti-cancer effects of aspirin have only recently become apparent, almost one hundred years after its launch.

The objectives of this work were:

- To detect heparanase gene expression in tissue and cell line (KATO-III) of SRCA and to assess its role in EMT and malignancy as in vitro.
- To analyze the induction of EMT of mesothelial cells as well as the regulation of neprilysin and modification of peritoneal cell surface in carcinomatosis patients
- To find whether thrombopoietin secreted by human ovarian cancer cells are active or not
- To report a case study, the presence of differentiated cells in ovarian carcinomatosis nodules after chemotherapy
- To evaluate EMT or differentiation in carcinomatosis as well as human cancer cell lines, breast cancer (MCF-7), ovarian cancer (OVCAR-3), gastric cancer (KATO III) into differentiated cells as in vitro and also a case study of ovarian cancer patient

MATERIALS AND METHODS

1. Biological samples and Drugs

1.1 Patient Ascites

Freshly-isolated ascites fluids from 14 cancer patients of the Hospital Lariboisière (Paris, France) were collected. As ascites fluids evacuation is part of the routine management of patients, only oral consent was obtained from them. Cells from ascites fluids were pelleted by a short spin at 3000rpm; for 10 minutes at 20 ^{0}C.

1.2 Patient tissue samples

Peritoneal membranes (ovarian cancer patient) and freshly-isolated ascites fluids (two ovarian and two gastric cancer patients) were obtained from General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France).

Tumor and corresponding normal gastric tissue specimens (SRCA tumoral, SRCA peri-tumoral, Non-SRCA tumoral and Non-SRCA peri-tumoral) were obtained from 21 patients with signet ring cell adenocarcinoma from General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France). Informed consent was obtained from each patient prior to surgery. All of the tumor and macroscopically normal gastric tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at −80°C until analysis. Tissue samples were histologically confirmed by hematoxylin and eosin staining.

We report the case of a female multipara patient of 85 years, who presented a pelvic mass of 10 cm wide painless axis associated with abdominal distension, nausea and ascites. The PET CT (positron emission tomography) and pelvic MRI (magnetic resonance imaging) confirmed the presence of a mixed primary ovarian tissue mass of about 10 cm and a large abdominal ascites with pleural effusion on right side and an important peritoneal carcinomatosis. Patient was evaluated as unoperatic in laparoscopy. Peritoneal biopsies were in favor of a poorly differentiated carcinoma, high-grade malignancy and evocative of a serosa nature.

After chemotherapy “Carbotaxol for a total of 7 treatments”, the marker CA 125 dropped from 3200 to 174 ng / ml and a regression of peritoneal carcinoma was observed. The patient was operated on for a cytoreduction associated with a posterior pelvectomy, protected omentectomy, appendectomy histero-ovarectomy, and lymph

node dissection. Tumors samples were obtained at diagnostic by biopsies and after chemotherapy, by radical surgeries of ovarian cancer patient. Stage of tumor categorized according International Federation of Gynecology and Obstetrics (FIGO) as malignant stage 4 serous adenocarcinoma.

1.3 Drugs

Drugs used in this study were: Suramin (Sigma Chemical Co, St. Louis, MO, USA) and aspirin (Aspegic, Sanofi-aventis, Paris, France).

1.4 Antibodies

The name, origin, clone name and dilution for each antibody used in this study

Antibodies	Origin	City	Country	Clone	Dilution
Ck AE1/AE3	Dako	Carpinteria - Californie	USA	AE1/AE3	1/50
NF	Dako	Glostrup	Denmark	2F11	1/100
SYP	Dako	Glostrup	Denmark	DAK-SYNAP	1/500
CHR-A	Dako	Glostrup	Denmark	DAK-A3	1/100
NSE	Dako	Glostrup	Denmark	BBS/NC/VI-H14	1/100
Ki67	Dako	Glostrup	Denmark	MIB-1	1/50
S100	Dako	Glostrup	Denmark	No clone	1/1000
CD56	Leica Biosystems	Benton	UK	1B6	1/50
WT-1	Dako	Carpinteria - Californie	USA	6F-H2	1/150

ER	Ventana	Tucson - Arizona	USA	Sp1	Not diluted
PR	Ventana	Tucson - Arizona	USA	1.00E+02	Not diluted
CD117 C-kit	Dako	Carpinteria - Californie	USA	No clone	1/50
β -catenin	BD-biosciences	Erembodegem	Belgium	14/β -catenin	1/50
CD3	Fisher scientific	Fremont	USA	Sp7	1/100
CD4	Ventana	Tucson - Arizona	USA	Sp35	Not diluted
CD8	Dako	Glostrup	USA	C8/144B	1/50
CD20	Dako	Glostrup	USA	CD20	1/50
E-cadherin	Dako	Glostrup	USA	NCH-38	1/50
Vimentin	Dako	Glostrup	USA	V9	1/500
Sox-2	Abcam	Cambridge	UK	57CT23.3.4	1/200
CD10	Leica Biosystems	Nanterre	France	NCL-L-CD10	1/10
CD31	Dako	Glostrup	USA	JC70A	1/20
D2-40	Dako	Glostrup	USA	D2-40	Predictive
Heparanase	Bioss	Greater Boston	USA	Polyclonal	1/50

2. Cell culture

2.1 Cell lines culture

Human cancer cell lines used were: Ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), colon (CT-26), lung (A549), leukemia (K562), cervical (HELA) and human microvascular endothelial (HMEC-1) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Normal human adult mesothelial cells (NMC) were purchased from Zen Bio, Inc. (Research Triangle Park, NC, USA).

2.2 Primary cell culture

Normal human adult mesothelial cells (NMC) were purchased from Zen Bio, Inc. (Research Triangle Park, NC, USA) and maintained in mesothelial cell growth medium (Zen-Bio, Inc.). Cells were harvested from the exponential growth phase and total RNA and protein were prepared as described below.

2.3 Ascites cell culture of patients

Ascitic fluids from patients were provided by the Digestive surgery department of Lariboisiere Hospital (Paris, France). All patients gave their written informed consent. Clinical and biological annotations were recorded in an Access database approved by the "*Commission Nationale de l'informatique et des Libertés*- France*"*. Medical records reported that 4 patients had cancer from ovarian origin. All patients were admitted for surgery during a period from October 2014 to February 2015. Each ascitic sample was centrifuged and the cell pellet obtained was cultured in flasks coated with 0.2% gelatin (Sigma, France) in DMEM medium (GIBCO, Saint Aubin, France) supplemented with 20% of heat-inactivated fetal bovine serum (FBS), 50ug/ml of streptomycin, 50 IU/ml of penicillin and 2nM of L-glutamine.

2.4 Conditioned media

Cells were seeded in plates or flasks, grown to 80% confluency and then incubated in serum-free culture medium. Three culture conditions were assayed : 1) in presence of Protein C (PC) (PROTEXEL, Courtaboeuf, France) at a concentration of 10 µg/ml, 2) in presence of activated Protein C (aPC) (Xigris, Suresnes, France) also at a concentration of 10 µg/ml, 3) without any addition as control. Cell flasks were incubated 5 hours prior to flow cytometry analysis and cells in plates were incubated

24 hours for co-culture experiments. Ba/F3 cell line was cultured in the presence of: 1) recombinant IL-3 (5ng/ml) (Sigma-Aldrich, Saint-Quentin Fallavier, France) 2) human recombinant thrombopoietin (TPO) (50 ng/ml) (LifeSpan Biosciences, USA) 3) PC (10 µg/ml) 4) aPC (10 µg/ml).

2.5 Co-culture of cancer cells and Ba/F3 cells

OVCAR-3, MDA-MB231 and K562 were cultured in bottom two cell culture compartments separated by a 0.4µm micropore membrane (Fisher Scientific, Illkirch, France) and Ba/F3 cells were cultured in the upper compartment.

3. Biological molecules

3.1 Extraction of total RNA

Gastric tissue specimens were homogenized with a polytron tissue homogenizer. Total RNA in cells and tissues was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction.

3.2 Reverse transcription

Total RNA in cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. RNA samples were transcribed to cDNA in a 20 µl volume, using the QuantiTect reverse transcription kit (Qiagen).

3.3 Polymerase chain reaction (PCR)

The thermal cycling comprised of the real time PCR following conditions: 95 ^{0}C for 5min, followed by 40 cycles (denaturation for15s at 95 ^{0}C, annealing for 20s at 60 ^{0}C) and extension for20s at 72 ^{0}C). The primers sequences and PCR product size for the target and reference gene are listed in Table 1.

mRNA expression levels of different markers were detected by real-time PCR with ß-actin as internal reference, using Mesa Blue qPCR Master Mix Plus for SYBR assay (Eurogentec) on the Mastercycler Realplex2 (Eppendorf).

Relative quantitation was calculated using the comparative threshold cycle (C_T) method with realplex software. Mean C_T of triplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (ß-actin) genes. The difference between the ΔC_T of the control experiment and the ΔC_T of each sample were calculated to give $\Delta\Delta C_T$. Fold increase in mRNA was calculated by $2^{-\Delta\Delta CT}$.

The PCR products of cell lines and tissues samples after real-time PCR were electrophoresed by E-Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific Inc, Waltham, USA).

3.4 Sequencing of nested-PCR products

Nested-PCR extracted DNA products were sequenced by Eurofins Genomic (Ebersberg, Germany), using the cycle sequencing technology (dideoxy chain termination / cycle sequencing) on ABI 3730XL sequencing machine. Sequences

were analyzed by the Basic Local Alignment Search Tool (BLAST) in the NCBI database.

Table-1

Gene	Primers (5`→3`)	Product length (bp)
Heparanase	AGACGGCTAAGATGCTGAAGAG	208
	TCTCCTAACCAGACCTTCTTGC	
FGF-2	CTGGCTATGAAGGAAGATGGA	149
	TGCCCAGTTCGTTTCAGTG	
TGF-β1	CAGAAATACAGCAACAATTCCTGG	186
	TTGCAGTGTGTTATCCCTGCTGTC	
VEGF-A	CCCACTGAGGAGTCCAACAT	173
	AAATGCTTTCTCCGCTCTGA	
E-Cadherin	TGGACAGGGAGGATTTTGAG	190
	ACCTGAGGCTTTGGATTCCT	
Snail	CCAATCGGAAGCCTAACTACAG	155
	GACAGAGTCCCAGATGAGCATT	
Slug	GCATTTCTTCACTCCGAAGC	151
	TGAATTCCATGCTCTTGCAG	
Vimentin	GAGAACTTTGCCGTTGAAGC	163
	GCTTCCTGTAGGTGGCAATC	
α-SMA	TTCAATGTCCCAGCCATGTA	222
	GAAGGAATAGCCACGCTCAG	
N-Cadherin	CAACTTGCCAGAAAACTCCAGG	205
	ATGAAACCGGGCTATCTGCTC	
Twist	GTCCGCAGTCTTACGAGGAG	159
	CCAGCTTGAGGGTCTGAATC	
Collagen-I	CCTGGATGCCATCAAAGTCT	153

	AATCCATCGGTCATGCTCTC	
Fibronectin	CCGAGGGACCTGGAAGTT	151
	ACTTGCTCCCAGGCACAG	
NANOG	ACCTTCCAATGTGGAGCAAC	199
	GAATTTGGCTGGAACTGCAT	
NESTIN	AACAGCGACGGAGGTCTCTA	220
	TTCTCTTGTCCCGCAGACTT	
CXCR-4	AGACCACAGTCATCCTCATCCT	190
	TAGAGGATGGGGTTCAGACAAC	
OCT3/4	GAAGGATGTGGTCCGAGTGT	183
	GTGAAGTGAGGGCTCCCATA	
SOX-2	GGGAAATGGGAGGGGTGCAAAAGAGG	151
	TTGCGTGAGTGTGGATGGGATTGGTG	
DNMT-3B	TGCTGCTCACAGGGCCCGATACTTC	242
	TCCTTTCGAGCTCAGTGCACCACAAAAC	
MRP-1	AGGTGGACCTGTTTCGTGAC	181
	CCTGTGATCCACCAGAAGGT	
MRP-2	GACCAACATTGTGGCTGTTG	163
	GAGGACCAGATCCAGCTCAG	
MRP-3	GGGCGTCTATGCTGCTTTAG	188
	CCTTGGAGAAGCAGTTCAGG	
MRP-4	AGAGCTGGTGCTCACTGGAT	154
	CGGTTACATTTCCTCCTCCA	
MRP-5	CCTTTTCACTCCCTCCATCA	185
	ACAGGTCTTGGAGCTGGAGA	
BCRP	CACCTTATTGGCCTCAGGAA	200
	CCTGCTTGGAAGGCTCTATG	
MDR-1	TGCCACCACGATAGCTGA	172

	CTGCTTCTGCCCACCACT	
LRP	GTGGAGGTCGTGGAGATCAT	186
	CCAAATCCAGAACCTCCTCA	
CD10	ACTGGGAGAAAACATTGCTGAT	183
	TTAACCGCATACTCTGGCCTAT	
MMP-2	ATGACAGCTGCACCACTGAG	174
	ATTTGTTGCCCAGGAAAGTG	
MMP-7	TGCTCACTTCGATGAGGATG	159
	TGGGGATCTCCATTTCCATA	
MMP-9	TTGACAGCGACAAGAAGTGG	174
	TCACGTCGTCCTTATGCAAG	
EPCR	CCTACAACCGCACTCGGTAT	181
	AAGATGCCTACAGCCACACC	
TF-3	GGGCTGACTTCAATCCATGT	195
	GAAGGTGCCCAGAATACCAA	
β-ACTIN	AGAGCTACGAGCTGCCTGAC	184
	AGCACTGTGTTGGCGTACAG	

4. Biochemical and analytical methods

4.1 Cell lines differentiation

To induce adipogenic, chondrogenic, osteogenic and neurocyte differentiation, confluent KATO-III, OVCAR-3 and MCF-7R were incubated for 14 days with StemPro®Adipogenesis, Chondrogenesis, Osteogenesis Differentiation Kit (GIBCO Life Technologies™) and Neurobasal® medium (Thermo Fisher Scientific). All induced cells were fixed for 30 min in 4% paraformaldehyde at room temperature and washed with phosphate-buffered saline (PBS). For assessment of calcium deposition in induced osteocytes, cells were with 2% Alizarin Red S solution (pH 4.2) for 2–3 minutes. The differentiated chondrocyte aggregates were stained with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 minutes and rinsed with distilled water to neutralize the acidity. The induced adipocytes were incubated with 60% isopropanol for 5 minutes and then stained with 60 % of oil red o (0.3g/ml isopropanol) in distilled water for 5 minutes. The induced neurocytes were stained with nissl staining solution (0.5g cresyl violet in 100ml of 0.6% glacial acetic acid) for 30minutes. An inverted microscope was used for imaging of all stained cells.

4.2 Immunohistochemistry

For anatomo-pathological analysis, the tissue samples (tumoral and peri-tumoral) and cell pellets (KATO-III) were dissected, fixed in PAF (4%) and embedded in paraffin. The slides (4 micron) were produced and colored by hematein-eosin-safran according to classical methods in the anatomo-pathogical laboratory. Similarly slides were also prepared using cytospin for ascitic cells of ovarian cancer. In parallel several slides were stained by antibodies coupled peroxidase using Benchmark Ultra apparatus (ROCHE VENTANA, Tucson, Arizona, USA) according their manufacturers. Proliferative index was evaluated according the sum of all cells in phase as metaphase, anaphase and telophase, counted in ten slid digital image on 40X (0.705 mm2) for each tissue slide. All antibodies and their origin and degree of dilution presented in Table 1. As controls, isotypic antibodies were used and the nuclei were labeled by hematoxylin bleuing (Ventana, Tuscan, USA). The pictures were taken by Leitz (Diaplan) microscopy with Nikon Cool pix 995 apparatus (Japan).

4.3 Immunofluorescence

KATO-III cells grown on multichamber slides were fixed at 25°C for 15 min with 4% paraformaldehyde in PBS, and then rinsed 3 times with PBS. Cells were permeabilized with 0.3% triton 15 min and rinsed 3 times with PBS at 25°C. The slide was incubated for 20 min with 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBS to block non- specific binding sites and then with anti HPA polyclonal coupled FITC (1:50 in 1% BSA; Bioss antibodies, Woburn, USA) overnight at 4 ° C, protected from light but in motion. After washing with PBS, the slide was again incubated with an anti-rabbit secondary antibody coupled to FITC (1/500 in 1% BSA) to maximize coloring for 1 hour at room temperature.

4.4 Cytokine array

We examined the gastric, colic and ovarian ascites as well as supernatant of mesothelial cells grown in 25% ovarian ascites using a protein cytokine array (RayBio ® Human Cytokine Antibody, Norcross, GA). This technique is based on the principle of sandwich immunoassay. It comprises essentially of screening, in duplicate, 174 different membrane-coupled anti-cytokines along with appropriate controls.

Mesothelial cells ($0.25x10^6$ cells per ml) were incubated in RPMI medium without fetal calf serum or within 25% ovarian ascites at 37˚C in a humidified atmosphere of 5% CO_2 for 24h. Supernatants containing cytokines were retrieved and the cytokines were allowed to couple with their specific antibodies previously immobilized on membranes. Membranes were saturated for 2h at room temperature with bovine serum albumin (BSA). Incubation of array membranes with supernatants was carried out overnight at 4˚C using corresponding antibodies. After several successive washes, membranes were incubated in the presence of a mixture of antibodies and anti-cytokines biotinylated at 4˚C overnight. Streptavidin, coupled with HRP, was added on the membranes for 2 h at room temperature. The presence of antibody coupled proteins was revealed by applying ECL (enhanced chemiluminescence) to the membranes, according to the recommendations of the manufacturer. Membranes were then exposed to photosensitive film (Kodak, X-OMAT, AR, USA).

The intensity of chemiluminescence captured on the photo- sensitive film was measured and recorded. After substracting the background noise, the results were expressed as a ratio of chemiluminescence intensity of experimental versus control spots.

4.5 Human Phosphokinase Array

A membrane-based antibody array (R&D Systems) that determines the relative levels of 45 different human phosphorylated protein kinases was utilized according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of KATO-III cell line treated with 0.2µg/ml recombinant human active heparanase protein (R&D Systems, Minneapolis, MN, USA) or 200µM Suramin (Sigma Chemical Co, St. Louis, MO, USA) or both into IMDM medium without FBS along with control for 5 hours were incubated overnight with the phosphokinase array membrane. The array was washed to remove unbound proteins followed by incubation with a mixture of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied to visualize the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound with densitometry by using a photosensitive film (Kodak, X-OMAT, AR, USA).

4.6 Evaluation of Heparanase and thrombopoietin by ELISA

After centrifugation of ascites fluid of 14 cancer patients (SRCA n=5, Non-SRCA n=3, Colic carcinoma n=6) at 1200rpm for 5 minutes at room temperature, the supernatant was separated. The heparanase was quantified using the commercially available HepAnalyze^{TM} Heparanase ELISA Kit (InSight Biopharmaceuticals Ltd. Rehovot, Israel) according to the manufacturer's instructions for ascitic supernatants. Briefly, 200 µl standard, blank or sample was plated in triplicate per well in a microtiter plate (96-well) coated with a monoclonal antibody specific to heparanase and incubated for 2 h at 37°C. Anti-heparanase monoclonal antibody conjugated to horseradish peroxidase substrate solution was added and incubated for 1.5hrs. Substrate was added to each well in turn and incubated in different environmental conditions. Stop solution was then added to each well when the first four wells containing the highest concentration of standards developed an obvious blue color. The optical density of each ELISA well was indicated by a microplate reader set to 450 nm. Accordingly, the heparanase value was calculated on the basis of the

'standard curve' drawn by the professional soft 'CurveExert 1.4'. The results were expressed in pg/ml.

After culture, the cells were carefully washed with phosphate-buffered saline (PBS) and then cells were cultured without fetal calf serum or additional growth factor in culture flask. After 18 hours, the cells were collected and soluble extracts were tested for TPO determination. The TPO was quantified using the commercially available ELISA (R & D Systems Quantikine Human TPO ELISA kit, Abingdon, UK), according to the manufacturer's instructions for cell culture supernatants. The results were expressed in pg/ml/1x10^6 cells.

4.7 Cell viability assay

Cell viability was assayed by Real Time-Glo™ MT Cell Viability Assay. In brief, KATO-III, (3×10^3/well) were seeded on 96-well plates, followed 24 h later by treatment with drugs; aspirin/suramin (or vehicle control) for 96 h. Confluent KATO-III (3×10^3/well), OVCAR-3 (5×10^2/well) and MCF-7R (10^3/well) were seeded on 96-well plates, followed 24 h later in inductor media (StemPro®Adipogenesis, Chondrogenesis, Osteogenesis Differentiation Kit and Neurobasal® medium) for 96 h. Mesothelial cells (5×10^2/well) were also seeded on 96-well plates, followed 24 h later by treatment with 25% gastric, colic or ovarian ascites for 48 h. Bioluminescence was measured with spectrofluorometer SAFAS Xenius XC. Cell viability was expressed as the percentage of absorbance of the drug-treated cells relative to that of the vehicle-treated cells. Each condition was done in triplicate. The experiment is representative of three independent experiences.

Ba/F3 cells were co-cultured separately with MDA-MB231 or OVCAR-3 or K562 cells, in the presence or absence of 10 µg/ml PC or aPC, as indicated above. Ba/F3 cells were cultured with IL-3, TPO, PC or aPC and served as control. After 72h, Ba/F3 cells were collected from each well and washed with PBS and then fixed with 4% (final concentration) formaldehyde. Then, Ba/F3 cells were suspended in cold binding buffer (10 mM HEPES pH 7.4, 140 mMNaCl, 2.5 mM CaCl2, 0.1% BSA), incubated for 15 min at 4°C with FITC conjugated Annexin-V (SouthernBiotech, Birmingham, USA) and shielded from light.

4.8 Wound healing assay

Mesothelial cells were grown (80% confluency) in 12 well plate with or without 25% ovarian ascites for 6h, 12h, 24h or 36h. The migration ability of mesothelial cells was evaluated by means of a scratch assay. A denuded area was generated on quiescent cell monolayers of mesothelial cells by scratching with a sterile pipette tip. The monolayer was washed twice with PBS and then incubated in medium having no FBS, with or without ovarian ascites. The cells were photographed at different time points. The width of the scratch was measured in three different places on the photograph to obtain a mean value, and migration was recorded as the difference (in micrometer) between the measurement at the baseline and after different intervals.

4.9 Fluorometric assays

A substrate based activity assay kit (Anaspec SensoLyte®, Belgium) that determines neprilysin activity in mesothelial cells was utilized according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of mesothelial cells grown in medium with or without 25% ascites for 6 days were used. Aliquots from each sample were incubated in the presence of the neprilysin substrate solutions for 60 min. The fluorescent product was measured in a spectrophotofluorometer (GloMax®-Multi Detection System, France) with excitation at 490 nm and emission at 520 nm.

4.10 Microcinematography

To document the kinetics of CT26 cell in the presence of fibrin, time-lapse microcinematography was done. Briefly, 100µl pool of human plasma with 2units of thrombin and 30µl of 0.025M $CaCl_2$ (Diagnostic STAGO, Parsippany, USA) was prepared in a 6-well plate. After 30-45 minutes, CT26 cells (5×10^5/well) were seeded on thrombin clot in medium. The plate was placed in the stagetop environmental chamber. Microcinematography was performed to acquire images every 2 minutes for 24 and 48hrs to study the migration behavior of CT26 in a temperature controlled room of 37 ^{0}C in a humidified atmosphere (>80%) containing 5% CO_2 by use of an EVOS® FL Auto Imaging System (Life Technologies™ Waltham, USA).

4.11 Scanning Electron Microscopy

Peritoneal membrane of a cancer patient was obtained from General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France). Informed consent was obtained from the patient prior to surgery. The membrane was fixed using 4% formaldehyde for 24hr and washed with 1x PBS buffer three times for each 5 min. After this, 2% glutaraldehyde was added on the membrane for 20 minutes and washed with 1x PBS buffer three times for each 5 min. After a final wash in distilled water, the samples were dehydrated in increasing concentrations of ethanol. Samples were dried by the critical point method with liquid CO2 and then sputter-coated with gold. They were observed with a S260 CAMBRIDGE scanning electron equipped with a LaB6 filament operating at 15kV and images were captured with the software "Orion" from (NCH Software).

4.12 Transmission Electron Microscopy

Primary ovarian cancer cells with fibrosis were detected using transmission electron microscopy. Briefly, primary cells after recovering from ascites were grown in DMEM medium. Cells (100 millions) were harvested, pelleted, fixed in 4% paraformaldehyde for 15 minutes. The samples were then rinsed with PBS (1X). Ultrathin sections (50–70 nm) were cut using a Leica RM2235 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and observed with a transmission electron microscope (Hitachi H-800; Hitachi, Tokyo, Japan).

4.13 FACS analysis

Confluent KATO-III, OVCAR-3 and MCF-7R ($0.1x10^6$ cells) were seeded in $25cm^2$ culture flask, followed 24 h later in control or inductor media (StemPro®Adipogenesis, Chondrogenesis, Osteogenesis Differentiation Kit and Neurobasal® medium) for 14 days.

The cells and tumor spheres were dissociated as single cell suspension, washed by PBS and then labeled with antibodies (10 µl/1×10^6 cells), including CD133 antibody (Miltenyi Biotec, Germany), mouse anti-human CD90 (BD Biosciences, San Jose, CA, USA), CD44, CD73, and CD117 at 4°C in the dark for 30 min.

OVCAR-3 and MDA-MB231 were cultured in flasks and incubated in a serum-free medium with or without PC/aPC stimulation as described above In half of the culture

medium was added 1µl/ml of protein transport inhibitor (containing Brefeldin A) provided by BD Biosciences (Le Pont de Claix, France). Then, cells were detached with accutase, washed with phosphate-buffered saline (PBS), and suspended in 100% heat-inactivated FBS. Cell fixation and permeabilization procedures were performed using the Perfix-nc assay kit as indicated by the manufacturer (Beckman Coulter, France). Cells were mixed at first with an anti-hTPO primary antibody (1:200) (Sigma-Aldrich, Saint-Quentin Fallavier, France), then washed with PBS and incubated with a fluorescein isothiocyanate (FITC) coupled secondary antibody (1:100). Cells were then washed twice and the pellet was re-suspended in 500 µl of buffer provided in the kit.

Flow cytometry was done using a standard Canto II eight-color flow cytometer (Becton Dickinson Biosciences, France) at 530 nm and data analysis was performed using Flowjo software (Flowjo LLC, USA).

4.14 Apoptosis assay

Apoptosis assay was performed by EdU staining was performed with a Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies™), according to the manufacturer's protocol. KATO-III cells line was synchronized with IMDM medium having 5% FBS for 24 h. The following day, cells were treated with or without 4.5mM aspirin and 200µM suramin. After four days of treatment, cells were trypsinized, harvested and incubated in culture medium with 15µM EdU for 2 hours. After incubation, cells were washed with 1% BSA in PBS and added 100µl of Click-iT fixative for 15 minutes at room temperature. After washing, cells were incubated in Click-iT Plus reaction cocktail including fluorescent dye (Alexa Fluor 647 picolyl azide) for 30 minutes. The flow cytometric cell analysis was performed, using a BD LSR II analytical flow cytometer (Becton Dickinson, San Jose, CA). MultiCycle AV (Phoenix Flow Systems) DNA analysis software enabled determination of the phase of cell cycle arrest by comparing percentages of each cell stage between the control and treatment groups (G1, S, G2/M).

RESULTS

Article No 1
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Neural Signature Expressed by Cells from Ovarian Carcinoma (A Case Report)

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Abstract

Aim: To demonstrate the presence of differentiated cells in ovarian carcinomatosis nodules after chemotherapy.

Patient and method: A patient of 85 years, who presented a pelvic mass of 10 cm. The anatomo-pathological study was performed on the biopsies (before treatment) and post operational samples (after treatment by Carbotaxol). Histological samples were analyzed with ovarian cancer markers for diagnosis. The immune cells (CD3, CD4, CD8 and CD20) and neural markers such as anti: neurofilament (NF), neural cell adhesion molecules NCAM (CD56), chromogranin A, neuronal specific enolase (NSE), S100 protein and synaptophysine were used for demonstrating the neuronal differentiation tendencies of carcinomatosis cells. Proliferation activities were studied by using proliferative index and Ki67 antibody.

Results: The histological result of biopsies of bilateral ovarian carcinomatosis showed the poorly differentiated monomorphic cell serous carcinoma (cytokeratin$^+$, estrogen receptor$^+$, protein S100$^+$, anti-wilms tumor-1$^+$ with proliferative index (31%) and high Ki67 marker (45%). Semi-quantitative histological evaluations of post operational samples presented two cellular quotas. One was composed of monomorphic cells with high proliferative index (19%) and ki67 marker (30%). In another quota, large size polymorphic cells with no proliferative index and Ki67 marker were distinguished. Before treatment, all neuronal markers except NSE and S100 protein were found negative in primary tumors. In the proliferative zone of post-operative samples, NSE and S100 protein markers persist with any other neuronal markers. These zones were highly infiltrated by CD3, CD4 and CD20 immune cells. In contrast, in degenerative non-proliferative zone, all primary tumor markers except Ki67, all neuronal markers except synaptophysine, and dramatically decreased infiltrated immune cells.

Conclusion: These results are in favors of differentiation of poorly differentiated ovarian cancer cells with high proliferative index to other tissue with no proliferation potential. Targeting of differentiation of cancer cells by differencing inductors may be a new way for cancer therapy.

Keywords: Anatomo-pathological study; Ovarian carcinomatosis; Neuronal markers; Immune cells; Cancer cell differentiation; NCAM (CD56); Chromogranin A; Neuronal specific enolase

Introduction

Ovarian carcinoma (OC) is the sixth most common malignancy in woman and the leading cause of death from gynecological cancer in the world [1]. Over 90% of malignant tumors are epithelial. It has been hypothesized that tumor can arise either from single layer of cells covering the ovary or from the epithelial lining of the fimbrial end of fallopian tube [2]. OC has a predisposition to metastatic involvement of the peritoneal cavity and form ovarian carcinomatosis nodules [3,4]. Late stage OC is characterized by widespread peritoneal dissemination, ascites and a high mortality rate with an overall survival ranging from 20 to 30% at 5 years after surgery [5].

Platinum associated to taxans chemotherapy is a standard treatment for ovarian cancer and has achieved a high response rate [6]. The development of drug-resistant cancer cells exhibiting the multidrug resistance phenotype is one of the major limitations of efficacy [5,7]. The mechanisms underlying chemoresistance in cancer are not clear. A growing amount of studies are inspiring the role of the cancer stem cells (CSC) in OC development [8]. Resistance to platinum-based chemotherapy is also associated with epithelial to mesenchymal transition in epithelial ovarian cancer [9,10]. A link between CSC and EMT has been suggested [11] and cancer cell can acquired differentiated phenotype after cytotoxic chemotherapy [12].

Previously, we reported that an ovarian cancer cell line OVCAR-3 NIH express both CD133 and CD117 stem cell markers and secret cytokines implicated in tumor growth and cell differentiation [13]. On the basis of pluripotency of cancer stem cell, the aim of this study is to demonstrate, co localization of ovarian carcinomatosis cells with high proliferative tendency and cells that expressed neural proteins without proliferative markers in peritoneal ovarian carcinoma.

Materials and Methods

Patient

We report the case of a female multipara patient of 85 years, who presented a pelvic mass of 10 cm wide painless axis associated with abdominal distension, nausea and ascites. The PET CT (positron

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emission tomography) and pelvic MRI (**magnetic resonance imaging**) confirmed the presence of a mixed primary ovarian tissue mass of about 10 cm and a large abdominal ascites with pleural effusion on right side and an important peritoneal carcinomatosis. Patient was evaluated as unoperatic in laparoscopy. Peritoneal biopsies were in favor of a poorly differentiated carcinoma, high-grade malignancy and evocative of a serosa nature.

After chemotherapy "Carbotaxol for a total of 7 treatments", the marker CA 125 dropped from 3200 to 174 ng / ml and a regression of peritoneal carcinoma was observed. The patient was operated on for a cytoreduction associated with a posterior pelvectomy, protected omentectomy, appendectomy histero-ovarectomy, and lymph node dissection. Tumors samples were obtained at diagnostic by biopsies and after chemotherapy, by radical surgeries of ovarian cancer patient. Stage of tumor categorized according International Federation of Gynecology and Obstetrics (FIGO) as malignant stage 4 serous adenocarcinoma.

Antibodies

16 antibodies (Table 1) as histopathological markers were used; cytokeratin (Ck AE1/AE3), neurofilament (NF), synaptophysine (SYP), chromogranin-A (CHR-A), neuronal specific enolase (NSE), proliferation marker (Ki67), neuronal protein S100 (S100), neural cell adhesion molecule (NCAM-CD56), wilms tumor-1 (WT-1), estrogen receptor (ER), progesterone receptor (PR), stem cell growth factor receptor CD117 C-kit and β-catenin). Immune cells were identified by anti:-CD3 (pan T cells), CD4 (T helper cells), CD8 (T suppressor cells) and CD20 (B cells).

Immunohistochemistry

Five nodules were extracted from patient and used for this study. For anatomo-pathological analysis, the samples were dissected, fixed in PAF (4%) and embedded in paraffin. The slides (4 micron) were produced and colored by hematein-eosin-safran according to classical methods in the anatomo-pathogical laboratory. In parallel several slides were stained by antibodies coupled peroxidase using Benchmark Ultra apparatus (ROCHE VENTANA, Tucson, Arizona, USA) according their manufacturers. Proliferative index was evaluated according the sum of all cells in phase as metaphase, anaphase and telophase, counted in ten slid digital image on 40X (0.705 mm^2) for each tissue slide.

All antibodies and their origin and degree of dilution presented in Table 1. As controls, isotypic antibodies were used and the nuclei were labeled by hematoxylin bleuing (Ventana, Tuscan, USA). The pictures were taken by Leitz (Diaplan) microscopy with Nikon Cool pix 995 apparatus (Japan).

Results

Anatomo-pathological studies

Figure 1A and 1B presents anatomo-pathologic aspect of tumor nodule before chemotherapy (biopsy). The tumor with high vascularization is poorly differentiated (1A). As presented in Figure 1B, only one quota made up of tumor cells of average size with a monomorphic cell distribution and high proliferative index (31%) without psammoma bodies.

Surgical biopsies of peritoneal nodules presented in Figure 2. The tumor nodule was well limited (2A). In all samples (n=5), the tumor nodule in addition of necrotic zone, is composed of two cellular quotas (2B and 2C). A quota made up of tumor cells of average size with a monomorphic (2D) with high proliferative index (19%). Another quota of tumor cells was observed as a large size polymorphic cells (2E) with almost no one proliferative index (<1%). All proliferative, non-differentiated zones are shown with black star and non-proliferative, degenerative with white star.

Immunohistochemistry analysis

Tissues markers: Immunohistochemistry analysis of all tissues, before and after cancer treatment using tissues markers presented in Table 2. Before treatment, all monomorphic proliferative cells express only CK (3A), Ki67 (3B), ER (3C) and WT-1 (3D) protein markers (Figure 3).

As presented in Figure 4, these cells express some cancer stem cell markers such as β-catenin (Figure 4A) and stem cell growth factor receptor CD117 (4B). Except S100 protein (4C) and NSE (4D), no pro-neuronal marker were detected such as NCAM (CD56) (Figure 5A), NF (5B), CHR-A (5C), and SYP (5D).

After treatment as presented in Figure 6, all ovarian cancer cell markers such as ER (6A and 6B), Ki67 (6C) and WT-1 (6D) were

Antibodies	Origin	City	Country	Clone	Dilution
Ck AE1/AE3	Dako	Carpinteria -Californie	USA	AE1/AE3	1/50
NF	Dako	Glostrup	Denmark	2F11	1/100
SYP	Dako	Glostrup	Denmark	DAK-SYNAP	1/500
CHR-A	Dako	Glostrup	Denmark	DAK-A3	1/100
NSE	Dako	Glostrup	Denmark	BBS/NC/VI-H14	1/100
Ki67	Dako	Glostrup	Denmark	MIB-1	1/50
S100	Dako	Glostrup	Denmark	No clone	1/1000
CD56	Leica Biosystems	Benton	UK	1B6	1/50
WT-1	Dako	Carpinteria -Californie	USA	6F-H2	1/150
ER	Ventana	Tucson - Arizona	USA	Sp1	Not diluted
PR	Ventana	Tucson - Arizona	USA	1E2	Not diluted
CD117 C-kit	Dako	Carpinteria -Californie	USA	No clone	1/50
β -catenin	BD-biosciences	Erembodegem	Belgium	14/β -catenin	1/50
CD3	Fisher scientific	Fremont	USA	Sp7	1/100
CD4	Ventana	Tucson - Arizona	USA	Sp35	Not diluted
CD8	Dako	Glostrup	USA	C8/144B	1/50
CD20	Dako	Glostrup	USA	CD20	1/50

Table 1: The name, origin, clone name and dilution for each antibody used in this study.

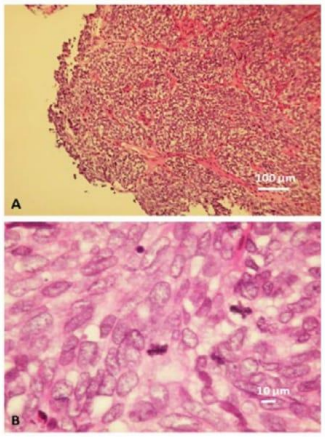

Figure 1: Anatomo-pathologic aspect of tumor nodule before chemotherapy (biopsy). The tumor is highly vascularized (A) and is poorly differentiated with a monomorphic cell distribution and high proliferative index (B).

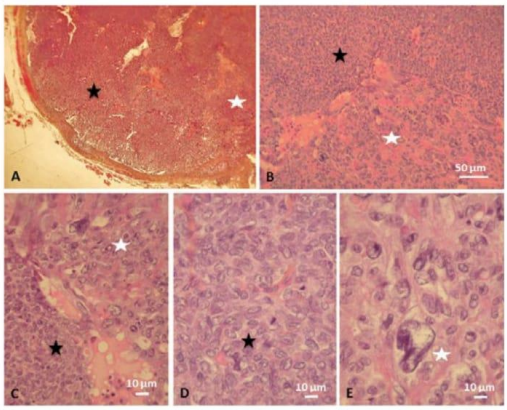

Figure 2: Anatomo-pathologic aspect of surgical biopsies of peritoneal nodules. The tumor nodule was well limited (2A) and composed of two cellular quotas (2B and 2C). A quota with proliferative monomorphic cells (2D) indicated with black star. Another quota with degenerative polymorphic cells (2E) indicated with white star.

detected in proliferative zone. CK, NSE, S100 and ER immunoreactivity was presented in both proliferative and non-proliferative regions (Figure 7 A, B and C). PR immunoreactivity was absent in all, pre and after chemotherapy (7D).

In contrast, in non-proliferative-degenerative zone, all neural markers studied in this study were present. In addition of NSE and S100 proteins, several pro neuronal markers as presented in Figure 8 such as NCAM (CD56) (8A), CHR-A(8B), NF(8C), except SYP (8D) were stained. As presented in Figure 6D, proliferative marker (Ki67) was stained predominantly in monomorphic proliferative zone. Any Ki67 immunoreactivity was not observed in degenerative zone. NF and CHR-A, markers of neuroendocrine tumors were detected only in some cells and also in non-proliferative degenerative zone (8B and C). After chemotherapy, the cell markers β-catenin persisted in all cells but CD117 C-kit not expressed (Figure 9).

Immune cells markers: Tumor infiltrative immune cells such as T cells (CD3) and their derivate cells, T helper (CD4), T suppressor (CD8) and B cells (CD20) were identified in surgical biopsies of peritoneal nodules after treatment. Significant differences were observed according the proliferative ($Ki67^{+}$) and non-proliferative ($Ki67^{-}$) zones. As presented in Figure 10. All degenerative zones were private from effective immune cells.

Discussion

In this case report, we demonstrated the neuronal signature in the nodule of peritoneal ovarian Carcinomatosis. Before treatment, tumor composed of small monomorphic cells with high score of mitosis and hyper vascularization. Numerous immune and inflammatory cells were identified in hematoxylin and eosin sections. This observation was confirmed by using anti: immune and inflammatory antibodies in immunocytochemistry.

Antibodies	Before Treatment		After Treatment	
	Proliferative zone	degenerative zone	Proliferative zone	degenerative zone
Ki67 (mitotic index)	+ (45%)	Non	+ (30%)	(<1%)
NSE	+	Non	+	+
Ck AE1/AE3	+	Non	+	+
WT-1	+	Non	+	+
β-catenin	+	Non	+	+
ER	+	Non	+	+
S100	+	Non	-	+
$CD117^{low}$	+	Non	-	-
NCAM-CD56	-	Non	-	+
NF	-	Non	-	+
CHR-A	-	Non	-	+
SYP	-	Non	-	-
PR	-	Non	-	-

Table 2: Comparison of immunohistochemical analysis of different tissue markers before and after cancer treatment.

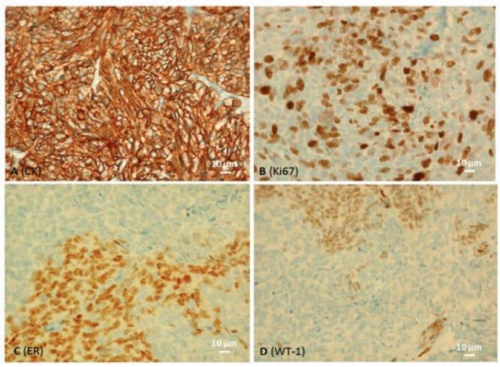

Figure 3: Immunohistochemistry analysis of ovarian cancer. Immunoreactivity with anti CK (A), Ki67 (B), ER (C) and WT-1 (D) was presented as protein markers in ovarian cancer (before treatment).

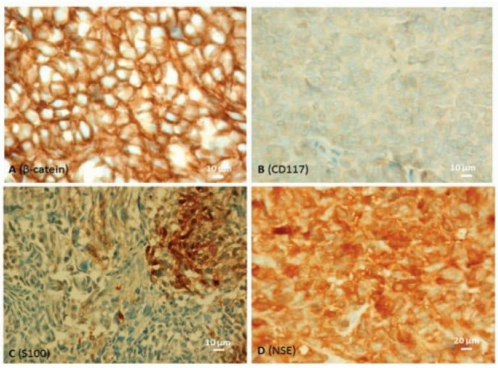

Figure 4: The expression of β-catenin (A), stem cell growth factor receptor CD117 (B), S100 protein (C) and NSE (D) were presented in ovarian cancer tissues (before treatment).

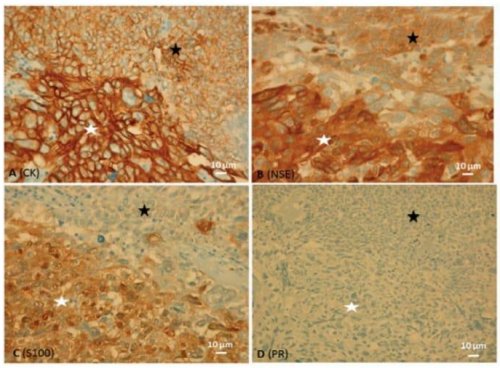

Figure 7: Presence of CK (A), NSE (B), S100 (C) in both proliferative and non-proliferative regions of ovarian tumor. Absence of PR Immunoreactivity (7D).

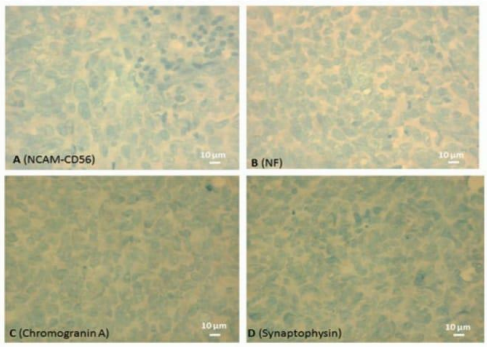

Figure 5: Absence of pro-neuronal marker, NCAM (CD56) (A), NF (B), CHR-A (C), and SYP (D) in ovarian cancer before treatment.

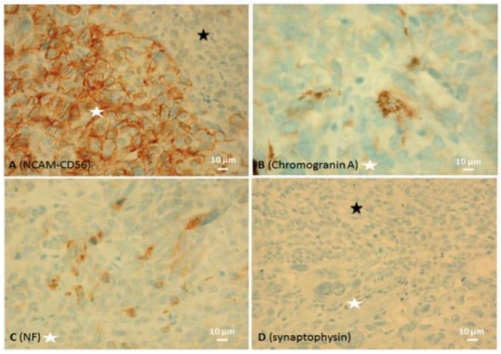

Figure 8: Presence of pro neuronal markers NCAM (CD56) (8A), CHR-A (8B), NF (8C), except SYP (8D) in degenerative zone of ovarian cancer after treatment.

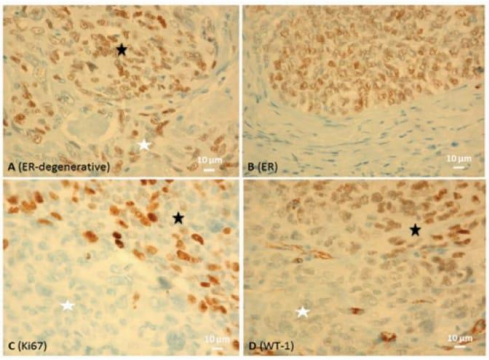

Figure 6: The presence of ovarian cancer cell markers ER in degenerative zone (A) and proliferative zone (B) and Ki67 (C) and WT-1 (D) in proliferative zone.

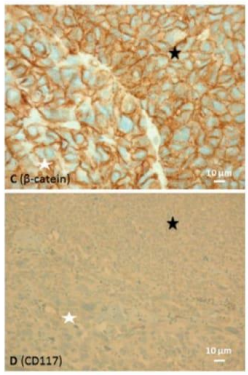

Figure 9: The presence of β-catenin (A) and absence of CD117 C-kit Immunoreactivity after treatment in both proliferative and degenerative zones.

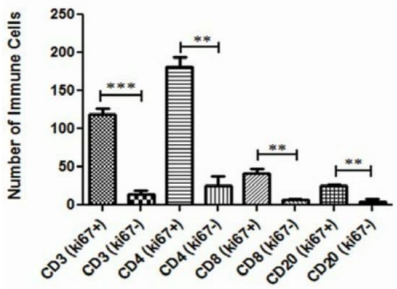

Figure 10: The distribution of immune cells CD3, CD4, CD8 and CD20 in both proliferative ($Ki67^+$) and degenerative ($Ki67^-$) zones.

After treatment, two zones were distinguished in all nodules; a zone with small monomorphic and proliferative cells and mitosis and the other non-proliferative zone without cell mitosis and absence of Ki67 marker. Curiously, all neural markers such as NSA, NCAM (CD56), S100 protein and CHR-A were found in non-proliferated zone. These results suggest that neuronal markers are associated with non-proliferative characteristic of ovarian cancer cells.

A consensus panel convened by the "American Association for Cancer Research" defined a cancer stem cell as "cell within a tumor that possesses the capacity to self–renew and to cause the heterogeneous lineage of cancer cells that comprise the tumor". Like normal stem cells, cancer stems cells were characterized by their radio and chemo resistance properties [14]. Differentiation of these stem cells can be benefic especially, when they differentiated into other cells. Induction of differentiation of human promyelocytic leukemia cell line (HL60) by retinoic acid was first demonstrated by Breitman et al. [15]. This finding suggests that the differencing agents can be used in therapeutic.

In this study we observed the expression of several neuronal markers in carcinomatosis nodules after the treatment of patients by chemotherapy. Belong to these; CD56 may be a crucial marker. CD56 antigen or NCAM is a glycoprotein expressed on the surface of neurons, glia and skeletal muscle. CD56 has been implicated in cell adhesion between them. Normal cells that stain positively for CD56 include NK cells, activated T cells, the brain and cerebellum, tissues and neuroendocrine. Tumors that are CD56-positive are myeloma, myeloid leukemia, neuroendocrine tumors, wilms tumor, neuroblastoma, lymphomas, NK / T cells, carcinoma of pancreatic acinar cells, pheochromocytoma, the paraganglioma, carcinoma small cell lung, and the family of Ewing sarcoma tumors. All this observation indicates ectopic localization of NCAM in different cells. Here, NCAM only expressed in non-proliferative quota of carcinomatosis nodule. In addition, some of these cells expressed NF and CHR-A. These results may suggest the differentiation of undifferentiated lineage cancer cells to other tissues with neuronal markers in carcinomatosis nodule after chemotherapy. Zueva et al. reported that 48.5% of the serous ovarian adenocarcinoma patient presents immunoreactivity with anti-NCAM antibodies [16].

NSE detected in ovarian cancer before and after treatment. The anti-NSE antibodies can be used to identify neuronal cell cells and with neuroendocrine differentiation. NSE is produced by pulmonary small carcinoma cells that are of neuroendocrine origin [11]. NSE is therefore a useful tumor marker for patients with lung cancer. In ovarian tumor, NSE as serum markers reported in ovarian immature teratoma and dysgerminoma. The presence of NSE in ovarian tumors may be in favor of neuronal differentiation tendencies.

Another neural signature is NF. It is a major corposant of neuronal cytoskeleton. Neurofilament light polypeptide as a novel hypermethylated gene associated with resistance to cisplatin-based chemotherapy [17]. In this study, the presence of NF positive cells in non-proliferative or dystrophic zone indicates that the ovarian carcinomatosis cells can be oriented to pre-neuronal cells. These results were confirmed by the presence of CHR-A in degenerative zone. CHR-A as a granin neuroendocrine secretory protein, is located in secretory vesicle of neuron and endocrine cells. CHR-A as well as NSE, NF and SYP were described in neuroendocrine tumors (carcinoid). In this study, in all nodules no cells were stained by SYP. Absence of mitosis and Ki67 index as well as lymph angiogenesis (tested by D2-40, Dako) (results not shown) in degenerative zone is in favor of non-carcinoid origin of the tumors.

Curiously, the amount of immune cells decreased dramatically in degenerative zone. These observations are in favors of lake of immunogenicity of cancer cells when they are differentiated to non-tumor cells. Over all, these results indicate that the poorly differentiated cancer cells can be switched in some stress condition such as chemotherapy to well differentiated cells. In this cell transition, proliferative cancer cells lake their mitotic activity and in consequence, all pathway signaling proteins implicated in this transition may be a target for cancer treatment. Future protocol using differencing inductors may be a new way for cancer therapies.

References

1. Siegel RL, Miller KD, Jemal A (2015) Cancer statistics. CA: A Cancer Journal for Clinicians 65: 5-29.
2. Nik NN, Vang R, Shih IM, Kurman RJ (2014) Origin and pathogenesis of pelvic (ovarian, tubal, and primary peritoneal) serous carcinoma. Annual Review of Pathology: Mechanisms of Disease 9: 27-45.
3. Lengyel E (2010) Ovarian cancer development and metastasis. The American journal of pathology 177: 1053-1064.
4. Seguin L, Desgrosellier JS, Weis SM, Cheresh DA (2015) Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance. Trends in cell biology 25: 234-240.
5. Pfisterer J, Ledermann JA (2006) Management of platinum-sensitive recurrent ovarian cancer. In: Seminars in oncology Elsevier 33: 12-16.
6. Johnson N, Miles T, Cornes P, Gilby E (2012) Taxane-platinum combination chemotherapy versus single agent platinum for the first-line treatment of epithelial ovarian cancer. The Cochrane Library.
7. Nath S, Daneshvar K, Roy L, Grover P, Kidiyoor A, et al. (2013) MUC1 induces drug resistance in pancreatic cancer cells via upregulation of multidrug resistance genes. Oncogenesis 2: e51.
8. Dyall S, Gayther SA, Dafou D (2011) Cancer stem cells and epithelial ovarian cancer. Journal of oncology 2010: 105269.
9. Marchini S, Fruscio R, Clivio L, Beltrame L, Porcu L, et al. (2013) Resistance to platinum-based chemotherapy is associated with epithelial to mesenchymal transition in epithelial ovarian cancer. European journal of cancer 49: 520-530.
10. Haslehurst AM, Koti M, Dharsee M, Nuin P, Evans K, et al. (2012) EMT transcription factors snail and slug directly contribute to cisplatin resistance in ovarian cancer. BMC cancer 12: 91.
11. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133: 704-715.
12. Brambilla E, Moro D, Gazzeri S, Brichon PY, Nagy-Mignotte H, et al. (1991) Cytotoxic chemotherapy induces cell differentiation in small-cell lung carcinoma. Journal of Clinical Oncology 9: 50-61.

13. Ducros E, Mirshahi S, Azzazene D, Camilleri-Broët S, Mery E, et al. (2012) Endothelial protein C receptor expressed by ovarian cancer cells as a possible biomarker of cancer onset. International journal of oncology 41: 433-440.

14. Abdullah LN, Chow EKH (2013) Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med 2: 3.

15. Breitman T, Selonick SE, Collins SJ (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proceedings of the National Academy of Sciences 77: 2936-2940.

16. Zueva E, Kairbayeva M, Nikogosyan S, Mozhenkova AV, Digaeva MA, et al. (2010) Immunological peculiarities of CD-56-positive serous ovarian adenocarcinoma. Bulletin of experimental biology and medicine 149: 604-608.

17. Chen B, Chen J, House MG, Cullen KJ, Nephew KP, et al. (2012) Role of neurofilament light polypeptide in head and neck cancer chemoresistance. Molecular Cancer Research 10: 305-315.

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Article No 2

The close relationship between heparanase and epithelial mesenchymal transition in gastric signet-ring cell adenocarcinoma

The close relationship between heparanase and epithelial mesenchymal transition in gastric signet-ring cell adenocarcinoma

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Running title: The role of heparanase in gastric signet-ring cell adenocarcinoma

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Abstract: Gastric signet ring cell adenocarcinoma (SRCA) is characterized by a remarkable fibrosis, rapid invasive progression, chemoresistance and metastasis to the peritoneum. Our objective was to analyze the role of heparanase (HPSE) gene expression in SRCA tissue. High expression of HPSE mRNA and protein was found in the tumor and in ascites of SRCA as well as in KATO-III cell lines. Beside of collagen-I, growth factors (TGF-β1 and VEGF-A, except FGF-2) and epithelial mesenchymal transition (EMT) markers (Snail, Slug, Vimentin, α-SMA and Fibronectin, except E-cadherin) were found higher in main nodule of SRCA as compared to peritumoral sites. Among MDR proteins, MDR-1 and LRP (lung resistance protein) were highly expressed in tumor cells. The formation of 3D cell spheroids was found to be correlated with their origin (adherent or non-adherent KATO-III). After treatment of KATO-III with a heparanase inhibitor (suramin), cell proliferation and EMT-related markers, besides collagen-1 expression, were down regulated. HPSE as well as EMT and stem cell markers were down regulated when KATO-III cells were incubated with stem cell differentiation-inducer selective medium. In conclusion, in SRCA, HPSE via an autocrine secretion is involved in acquisition of mesenchymal phenotype and tumor cell malignancy.

Keywords: Signet ring cell adenocarcinoma, heparanase, epithelial mesenchymal transition.

Introduction

Gastric signet ring cell adenocarcinoma (SRCA) is characterized by the presence of isolated or small groups of malignant non-cohesive cells (>50%) containing intracytoplasmic mucin and exhibits diffuse growth and invasion without forming ducts [614]. Peritoneal invasion is the most frequent type of metastasis in patients with SRCA. They frequently occur at the later stages of gastric carcinoma, especially after surgery and significantly contributes to gastric cancer-related mortality [615]. One of the characteristics of SRCA is its resistance to chemotherapy. Most tumor cells with multidrug resistance are characterized by the overexpression of multidrug resistance molecules such as P-glycoprotein (P-gp), lung resistance protein (LRP) and multidrug resistance-associated protein (MRP)[616, 617]. Another mechanism of chemoresistance is the epithelial-mesenchymal transition (EMT) [618]. EMT plays an essential role in tumor development namely cancer metastasis [73, 186, 619, 620] and fibrosis [621]. During EMT, cells display on one hand decreased expression of epithelial cell markers such as E-cadherin and on the other hand increased expression of mesenchymal cell markers (Snail, Slug, Vimentin, α-SMA, fibronectin, collagen-I) and enhanced cell motility[73, 186, 619-621]. SRCA was also characterized by the presence of tissues fibrosis [622]. EMT associated with hypersecretion of heparanase was reported in renal fibrosis [507]. Heparanase (HPSE) as a multitasking protein, characterized by enzymatic and non-enzymatic activities, modulates TGF-β-induced EMT and fibrosis [623, 624]. Enzymatically active HPSE binds to the cation-independent mannose 6-phosphate receptor (CD222) expressed on cell surfaces to degrade extracellular matrix [625] with heparan sulfate, a side chain of heparin sulfate proteoglycans (HSPGs) [626]. HSPGs store various cytokines and growth factors such as basic fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), interferon-β (INF-β), and transforming growth factor-β (TGF-β)[465, 627, 628]. HPSE has also non-enzymatic functions [629]. The signaling activity is achieved by interacting with transmembrane proteins, leading to an activation of Akt and Src [630], or modulating the activity of factors such as FGF-2 and TGF-β [506, 507]. Recent analysis showed that degradation of heparan sulfate by HPSE increases permeability across the basal membrane and, thus, stimulates the release of cytokines and growth factors leading to tumor development via angiogenesis and metastasis [465, 628, 631]. Recently, protein or messenger RNA (mRNA) expression of HPSE

has been identified in various cancer cells, and the overexpression of HPSE protein or mRNA in tumor cells has been reported and correlated with the metastatic potential of tumor cells *in vitro* and *in vivo* as well as with poor prognosis[440, 475, 632, 633]. However its role in SRCA is still not clearly clarified. The aim of this study was to identify the position of heparanase in the SRCA malignancy.

Materials and Methods

Materials

Cell lines and reagents

Human cancer cell lines used were: Ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), leukemia (K562), cervical (HELA) and human microvascular endothelial (HMEC-1) and were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Ascitic fluids from 14 cancer patients of the Hospital Lariboisière (Paris, France) were collected. As ascitic fluid evacuation is part of the routine management of patients, only oral consent was obtained from them. Cells from ascitic fluids were pelleted by a short spin at 1000 rpm and the supernatant was collected after a 10 min centrifugation.

Drug used in this study was: Suramin (Sigma Chemical Co, St. Louis, MO, USA).

Culture

Cells were cultured in RPMI 1640 medium or IMDM medium containing 10% heat-inactivated fetal bovine serum (FBS), 50ug/ml of streptomycin, 50 IU/ml of penicillin and 2nM of L-glutamine (Gibco, Saint Aubin, France). Cells were incubated at 37^0C in a humidified atmosphere containing 5% CO_2.

Tissues

Tumor and corresponding normal gastric tissue specimen (SRCA tumoral, SRCA peri-tumoral, Non-SRCA tumoral and Non-SRCA peri-tumoral) were obtained from 21 patients with signet ring cell adenocarcinoma from the General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France). Informed consent was obtained from each patient prior to surgery. All of the tumor and macroscopically normal gastric tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at −80°C until analysis. Tissue samples were histologically confirmed by hematoxylin and eosin staining.

Evaluation of HPSE by ELISA: After centrifugation of ascitic fluid of 14 cancer patients (SRCA n=5, Non-SRCA n=3, Colic carcinoma n=6) at 1200 rpm for 5 minutes at room temperature, the supernatant was collected. The HPSE was quantified using the commercially available HepAnalyze™ HPSE ELISA Kit (InSight Biopharmaceuticals Ltd. Rehovot, Israel) according to the manufacturer's instructions. The results were expressed in pg/ml.

Immunocytochemistry

KATO-III cells grown on multichamber slides were fixed at 25°C for 15 min with 4% paraformaldehyde in PBS, and then rinsed 3 times with PBS. Cells were permeabilized with 0.3% triton 15 min and rinsed 3 times with PBS at 25°C. The slide was incubated for 20 min with 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBS to block non- specific binding sites and then with anti HPSE polyclonal (1:50 in 1% BSA; Bioss antibodies, Woburn, USA) overnight at 4 °C, protected from light. After washing with PBS, the slides were further incubated with a secondary anti- Ig rabbit antibody coupled to FITC (1/500 in 1% BSA) to maximize coloring, for 1 hour at room temperature

Fluorometric assays

A substrate based activity assay kit (Kaivogen, Turku, Finland) that determines heparanase activity in term of fondaparinux® degradation was utilized according to the manufacturer's instructions. Briefly, various cancer cell lines (10^6 cancer cells) ovarian (OVCAR-3), lung (A549) and gastric (KATO-III) were incubated in serum-free culture medium at 37˚C in a humidified atmosphere of 5% CO2 for 24hrs. 15 µl of each supernatant diluted to one half of fondaparinux (50 µg/ml) with 135 µl of buffer pH 5.2 were incubated for 2 hrs. at 37 °C. Residual activity of fondaprinux was then evaluated by adding factor Xa and its substrate in each supernatant mixture using STA Compact Max® (Gennevilliers, France) with excitation at 490 nm and emission at 520 nm. Results are expressed in µg/ml.

Cell viability assay

Cell viability was assayed by Real Time-Glo™ MT Cell Viability Assay. In brief, cells (3×10^3/well) were seeded on 96-well plates, followed 24 h later by treatment with drugs (or vehicle control) for 96 h. Bioluminescence was measured with the

spectrofluorometer SAFAS Xenius XC. Cell viability was expressed as the percentage of absorbance of the drug-treated cells relative to that of the vehicle-treated cells.

RNA isolation, RT and real-time PCR

Gastric tissue specimens were homogenized with a polytron tissue homogenizer. Total RNA in cells and tissues was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. RNA samples were transcribed to cDNA in a 20 µl volume, using the QuantiTect reverse transcription kit (Qiagen).

The thermal cycling comprised of the real time PCR as per following conditions: 95 ^{0}C for 5 min, followed by 40 cycles (denaturation for 15 sec at 95 ^{0}C, annealing for 20 sec at 60 ^{0}C and extension for 20 sec at 72 ^{0}C). The primer sequences and PCR product size for the target and reference genes are listed in complementary results Table 1.

mRNA expression levels of different markers were detected by real-time PCR with ß-actin as internal reference, using Mesa Blue qPCR Master Mix Plus for SYBR assay (Eurogentec) on the Mastercycler Realplex2 (Eppendorf).

Relative quantitation was calculated using the comparative threshold cycle (C_T) method with realplex software. Mean C_T of triplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (ß-actin) genes. The difference between the ΔC_T of the control experiment (Kato III) and the ΔC_T of each sample were calculated to give $\Delta\Delta C_T$. Fold increase in mRNA was calculated by $2^{-\Delta\Delta CT}$.

The PCR products of cell lines and tissue samples after real-time PCR were electrophoresed by E-Gel Precast Agarose Electrophoresis System.

Human Phosphokinase Array

A membrane-based antibody array (R&D Systems, Raffles, China) that determines the relative levels of 45 different human phosphorylated protein kinases was used according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of KATO-III cell line treated with or without 200µM Suramin (Sigma Chemical Co, St. Louis, MO, USA) into IMDM medium without FBS along with control for 5 hours

were incubated overnight with the phosphokinase array membrane. The array was washed to remove unbound proteins followed by incubation with a mixture of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied to visualize the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound with densitometry by using a photosensitive film (Kodak, X-OMAT, AR, USA).

Analysis of cell cycle in KATO-III after treatment with suramin

Apoptosis assay was performed by EdU staining using a Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies), according to the manufacturer's protocol. KATO-III cell line was synchronized with IMDM medium having 5% FBS for 24 h. The following day, cells were incubated for 4 days with or without 200µM suramin and then trypsinized. These harvested cells were incubated in culture medium with 15µM EdU for 2 hours. After incubation, cells were washed with 1% BSA in PBS and 100µl of Click-iT fixative was added for 15 minutes at room temperature. After washing, cells were incubated in Click-iT Plus reaction cocktail including fluorescent dye (Alexa Fluor 647 picolyl azide) for 30 minutes. The flow cytometric cell analysis was performed, using a BD LSR II analytical flow cytometer (Becton Dickinson, San Jose, CA). MultiCycle AV (Phoenix Flow Systems) DNA analysis software enabled determination of the phase of cell cycle arrest by comparing percentages of each cell stage between the control and treatment groups (G1, S, G2/M).

Differentiation of KATO-III cell line

To induce adipogenic, chondrogenic, osteogenic and neurocyte differentiation, confluent KATO-III were incubated for 14 days with StemPro®Adipogenesis, Chondrogenesis, Osteogenesis Differentiation Kit (GIBCO Life Technologies™) and Neurobasal® medium (Thermo Fisher Scientific). All induced cells were fixed for 30 min in 4% paraformaldehyde at room temperature and washed with phosphate-buffered saline (PBS). For assessment of calcium deposition in induced osteocytes, cells were treated with 2% Alizarin Red S solution (pH 4.2) for 2–3 minutes. The differentiated chondrocyte aggregates were stained with 1% Alcian blue solution prepared in 0.1 N HCL, for 30 minutes and rinsed with distilled water to neutralize

the acidity. The induced adipocytes were incubated with 60% isopropanol for 5 minutes and then stained with 60 % of oil red O (0.3g/ml isopropanol) in distilled water for 5 minutes. The induced neurocytes were stained with cresyl violet solution (0.5g cresyl violet in 100ml of 0.6% glacial acetic acid) for 30minutes. An inverted microscope was used for imaging of all stained cells.

Results:

1- Gastric signet ring cell adenocarcinoma nodules express heparanase

As presented in figure 1A, high expression (*p=0.0327*) of HPSE was found in the ascitic samples of primary signet ring cell adenocarcinoma (SRCA) of stomach as compared to non-SRCA of stomach and colic cancer by ELISA**.** Figure 1B shows that mRNA of HPSE is significantly higher in SRCA than non-SRCA stomach cancer (*p=0.0002*). HPSE mRNA was also expressed in peritumoral zone of SRCA. As presented in figure 1C, HPSE mRNA was found to be more (p=0.015) in peritumoral-SRCA compared to peritumoral non-SRCA. Relative expression of HPSE by several cancer cells such as primary SRCA cells isolated from peritoneal fluid of SRCA patients and various cell lines such as ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), leukemia (K562), cervical (HELA), human microvascular endothelial (HMEC-1) cell lines presented in figure 1D. Relative heparanase activity in supernatants of various cancer cell line such as ovarian (OVCAR-3), lung (A549), gastric (KATO-III) were presented in figure 1E. The presence of heparanase was confirmed by immunohistochemistry in KATO III cell line (Figure 1F). These results are in favor of high expression of HPSE mRNA as found by qPCR in SRCA.

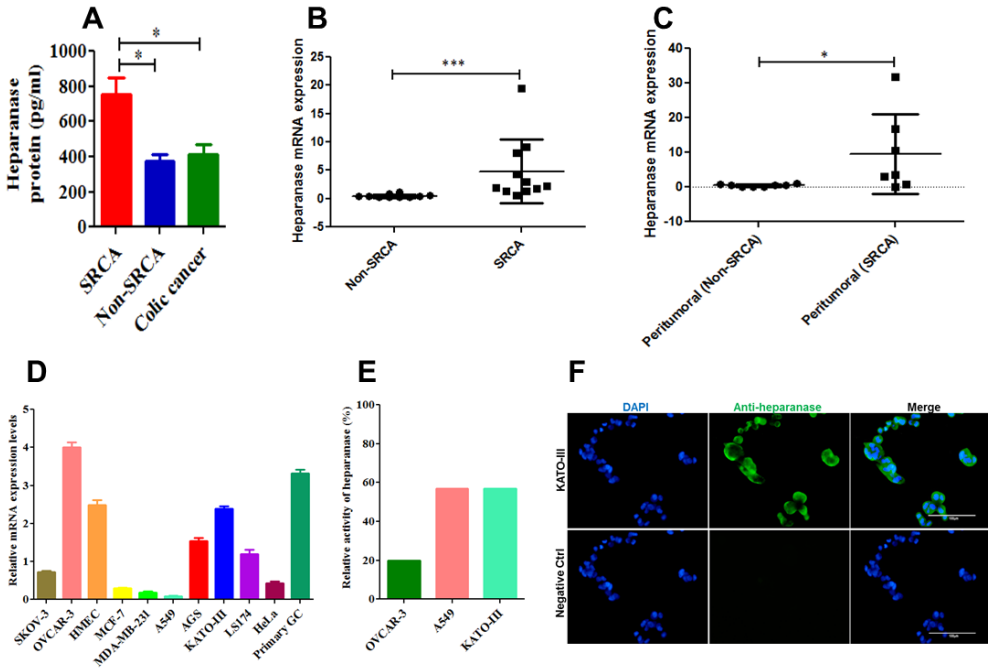

Figure 1: mRNA and protein expression of heparanase in clinical samples and cell lines including KATO-III

Heparanase protein was found in the ascitic samples of primary signet ring cell adenocarcinoma (SRCA) of stomach as compared to Non-SRCA of stomach and colic cancer by ELISA (SRCA n=5, Non-SRCA n=3 and colic cancer n=6) (A) mRNA expression of heparanase was found higher in SRCA (n=11) than non-SRCA (n=10) (B) as well as in peritumoral-SRCA (n=7) than peritumoral non-SRCA (n=8) (C) Heparanase gene expressed by various cell lines ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), leukemia (K562), cervical (HELA), human microvascular endothelial (HMEC-1) cell lines and primary SRCA (Primary GC) via RT-PCR, (D). The mean of three experiments for heparanase activity (evaluated by degradation of fondaparinux at pH 5) observed in supernatants of various cancer cell lines such as ovarian (OVCAR-3), lung (A549) and gastric (KATO-III),(E). Heparanase protein expression level in KATO-III by immunofluorescence is shown (F) The results are expressed as mean ± SEM of three independent experiments *P<0.05, ***P<0.001, statistically significant.

2- Gastric signet ring cell adenocarcinoma express EMT and multi drug resistance markers

Figure 2A present the relative mRNA expression of several growth factors such as FGF-2, TGF-β1 and VEGF-A as well as EMT markers (E-cadherin, Snail, Slug, Vimentin, α-SMA and fibronectin) in tumoral tissues of SRCA as compared with the peripheral region of tumor. Except E- Cadherin and FGF-2, all other markers tested were expressed highly in tumor tissues as compared with periphery of tumors. These results suggest mesenchymal characteristics of tumor tissues ~~in~~ and are also in favor of drug resistance in SRCA's patients. When the drug transporter (MDR-1 (Pg-1) , MDR-2, MDR-3, MDR-4, MDR-5, BCRP, MDR-1 and LRP) mRNA expression in tumor region, determined by RT-qPCR, as presented in figure 2B**,** all ATP-binding cassette proteins as well as lung resistant protein were detected. However, ~~a~~among these drug transporters, MDR-1 and LRP were the most expressed

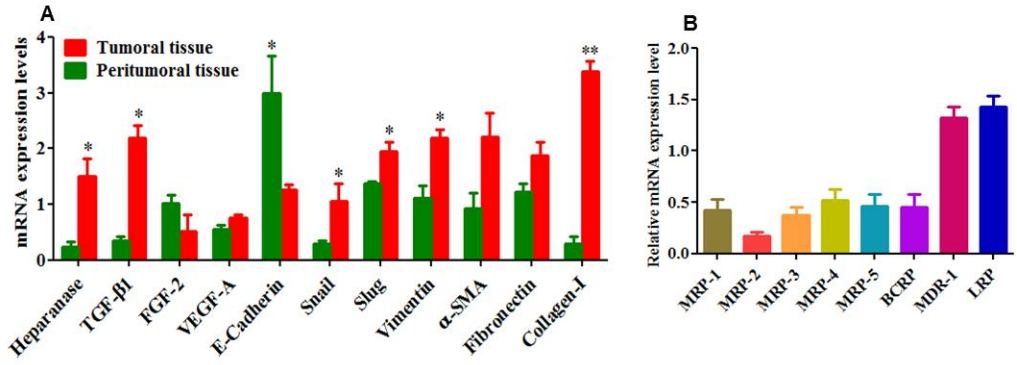

Figure 2: mRNA expression of heparanase, growth factors, EMT markers and drug transporters in clinical samples

Heparanase, growth factors (TGF-β1 and VEGF-A) except FGF-2 and epithelial marker like E-cadherin were found higher while mesenchymal markers (Snail, Slug, Vimentin, α-SMA and fibronectin) were lower in tumoral tissue of SRCA as compared to peritumoral tissue by qPCR **(A).** Similarly, of all the drug transporters (MDR-1, MDR-2, MDR-3, MDR-4, MDR-5, BCRP, MDR-1 and LRP) **o**nly two (MDR-1 and LRP) were found higher in SRCA tissue samples **(B).** The results are expressed as mean ± SEM of six independent experiments *P<0.05, **P<0.01, statistically significant.

3- KATO III cell line formed spheroid clusters and expressed EMT markers *in vitro*

In culture medium, KATO III cell line has two phenotypes; adherent and non-adherent cells (figure 3A) and formed the spheroids. As presented in figure 3B micro-cinematographic studies of these cell clusters indicated that their diameters varied according to their origin (adherent and non-adherent cells). As presented in figure 3C, the non-adherent cells are more spherogenic than adherent cells ($p = 0.0001$). Gene expression of EMT markers in both adherent and non-adherent KATO-III cells was studied. No alteration in gene expression ($p=0.124$) of epithelial-mesenchymal transition (EMT)-related molecules was observed when adherent and non-adherent KATO-III cells were grown separately for one week (Table 1).

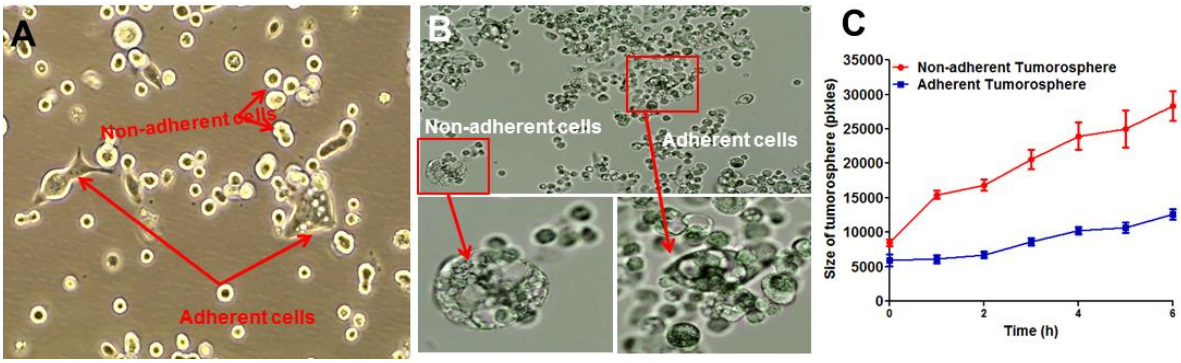

Figure 3: Spheroid cluster formation in both adherent and non-adherent KATO-III cell line.

Adherent and non-adherent cells of KATOIII **(A)** Tumorosphere cells derived from adherent and non-adherent KATO-III **(B)** quantification of the maximal tumorosphere outgrowth diameter (pixels) during 6hrs **(C).**

4- Suramin, an heparanase inhibitor down regulated TGF-β and collagen-1 mRNA KATO-III cells .

As presented in figure 4, when KATO-III cells were cultured in a medium containing suramin, there is a down regulation of TGFβ-1 and collagen-I expression in SRCA cell line in a time dependent manner. This result suggests that suramin simultaneously down regulates TGFβ-1 and collagen-1 expression in KATO-III cells.

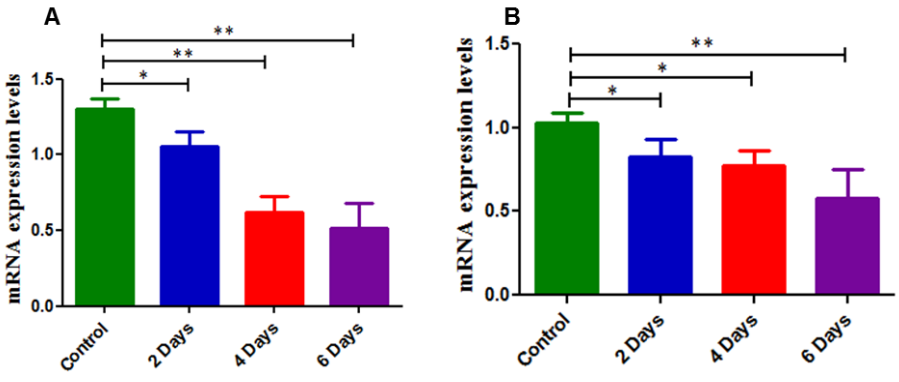

Figure 4: mRNA gene expression of TGFβ-1 and collagen-I in KATO-III cell line after treatment with suramin.

Suramin lowers the expression of TGFβ-1 **(A)** and collagen-I **(B)** in KATO-III in a time dependent manner by qPCR. The results are expressed as mean ± SEM of six independent experiments *P<0.05, **P<0.01, statistically significant.

5- Suramin down regulates EMT and stem cell markers as well as inhibits cell cycle and proliferation of KATO III cell line

As presented in figure 5A, incubation of KATO III cell line in a culture medium with suramin, down regulates heparanase expression in SRCA cell line in a time dependent manner. After 6 days incubation of the cells with suramin, mesenchymal markers such as Slug, Vimentin and α-SMA are significantly decreased (*p= 0.005*) while the E-cadherin is up regulated (*p= 0.01*) (Figure 5B). Suramin also decreased (Figure 5C) the expression of stem cell marker CXCR4, OCT3/4 and NANOG (*p= 0.005*). The inhibition of KATO-III cell proliferation observed when these cells were incubated with suramin (*p= 0.005*) (Figure 5D). The influence of suramin on cell cycle (G1, M and G2) of KATO-III is presented in figure 5E. The percentage of cells in each phase of the cell cycle is presented in figure 3F). These results indicate that suramin via phase S pathway inhibits cancer cell proliferation.

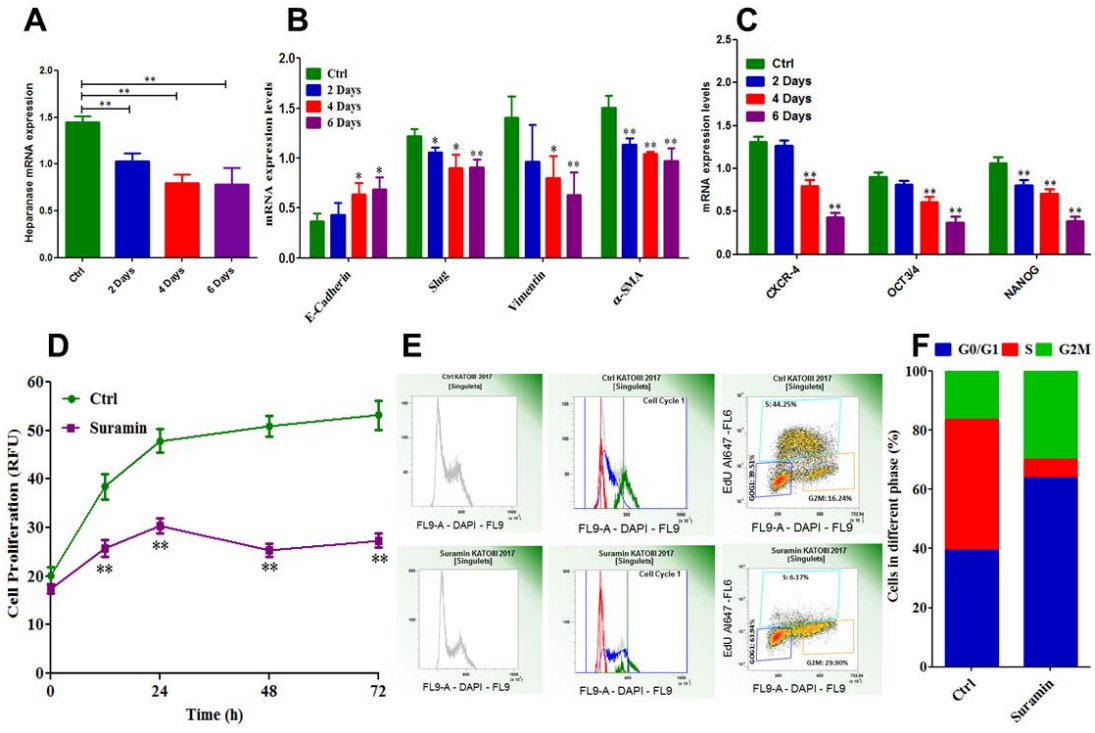

Figure 5: mRNA expression of EMT markers, stem cell markers as well as cell cycle arrest and cell proliferation in KATO-III after treatment with suramin.

Expression of heparanase **(A),** mesenchymal markers (Slug, Vimentin and α-SMA) **(B)** and stem cell markers (CXCR-4, OCT3/4 and NANOG) **(C)** were found lower while epithelial marker (E-cadherin) was found higher **(B)** in KATO-III after treatment with suramin by qPCR**.** Suramin inhibits the proliferation **(D)** and cell cycle arrest **(E)** in KATO-III after treatment. The data indicates the percentage of cells in each phase of the cell cycle **(F)**. The results are expressed as mean ± SEM of six independent experiments (*P<0.05, **P<0.01) and are statistically significant.

6- Cell inducer differentiation medium down regulates heparanase and stem cell marker expression as well as inhibits cell proliferation in KATO III cell line

When SRCA cell line was incubated with different culture medium targeted for stem cell differentiation in adipocytes, chondrocytes, osteocytes and neuronal cells, the expression of heparanase significantly decreased as seen in adipocytes ($p= 0.005$), chondrocytes ($p= 0.005$), osteocytes ($p= 0.006$) (figure 6A). This phenomenon associated with a high expression of E-cadherin ($p= 0.005$) and down regulation ($p= 0.006$) of stem cell markers such as Slug, vimentin and α-SMA mRNAs (figure 6B) as well as inhibition ($p= 0.002$) of KATO cell proliferation (figure 6C). Stem cell markers (CD90 and CD117) were found to be reduced after cell differentiation **(**Figure 6D**).** Adipogenic, chondrogenic, osteogenic and neurogenic differentiation of KATO-III, confirmed by the visualization of intracytoplasmic lipid drops stained in red using Oil Red O, a blue coloration due to proteoglycan synthesis using Alcian blue, and a red coloration due to extracellular calcium deposits using Alizarin Red S and dark black-violet due to extensive somata-associated accumulations of nissl bodies respectively (Figure 6E). These results confirm the influence of selective medium on the heparanase expression and indicate that stem cell and mesenchymal characteristics of KATO III cell line.

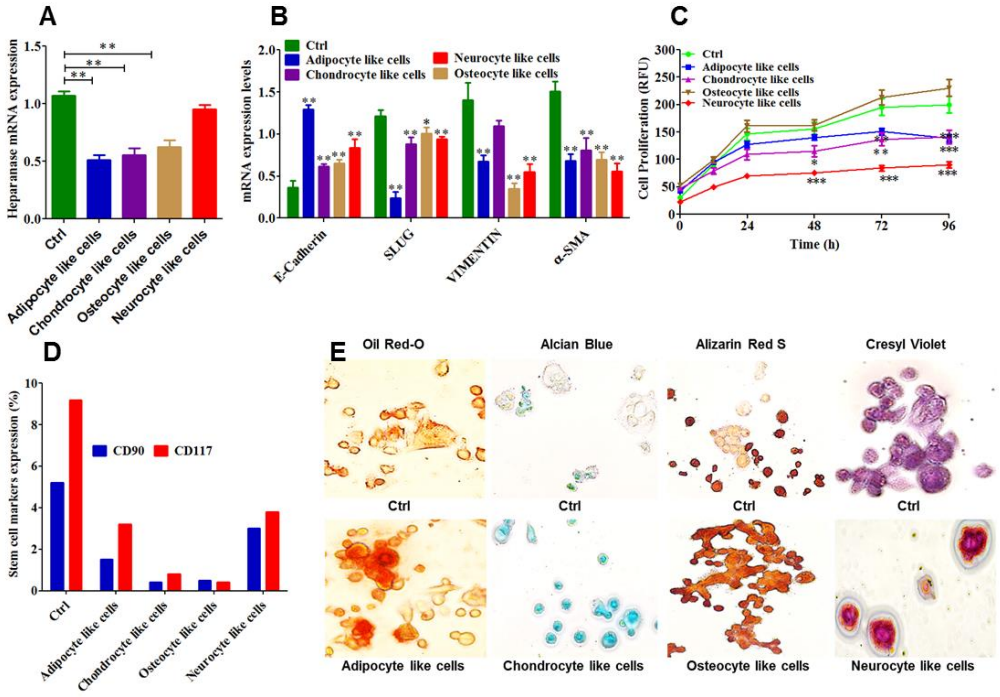

Figure 6: mRNA expression of heparanase and EMT markers ascertained by qPCR, stem cell markers by flow cytometry, cell proliferation and coloration of differentiated KATO-III cells after induction

Expression of heparanase **(A)** and mesenchymal markers (Slug, Vimentin and α-SMA) were found lower while the epithelial marker (E-cadherin) was found higher **(B)** in KATO-III by qPCR after inducing differentiation. Inductor media inhibited the proliferation of induced differentiated cells **(C)**. Stem cell markers (CD90 and CD117) were also found to be lower after differentiation via flow cytometry **(D).** Adipogenic, chondrogenic, osteogenic and neurogenic differentiation of KATO-III was confirmed by coloration **(E).**

7- Suramin modified phosphokinase activity pattern of KATO-III cells .

As presented above, Kato III cell line, in an autocrine manner or as a matter of fact by a paracrine pathway, secretes heparanase and influences endogenous kinase activity. The base line of phosphokinase activity of KATO-III cells (mean of three experiments) grown in the serum free culture medium presented is in figure 7A. These results indicate that some phosphokinase pathways such as GSK-3α/β, β-catenin, Chk-2, AMPKα-1, PRAS40 and C-Jun (more expressed proteins presented) are active in these cells. This pattern was modified when the cells were incubated in culture medium with the suramin. Results presented in figure 7B indicate that 6 different kinases were altered in KATO-III treated for 5 hours as compared to the

control. After treatment with suramin, two of the markers were upregulated (HSP60 and C-Jun) whereas 4 (GSK-3α/β, β-catenin, Chk-2 and AMPKα-1), were down regulated. These results correspond to the inhibition of secretion of non-activated heparanase and indicate the crucial role of heparanase in cell hemostasis.

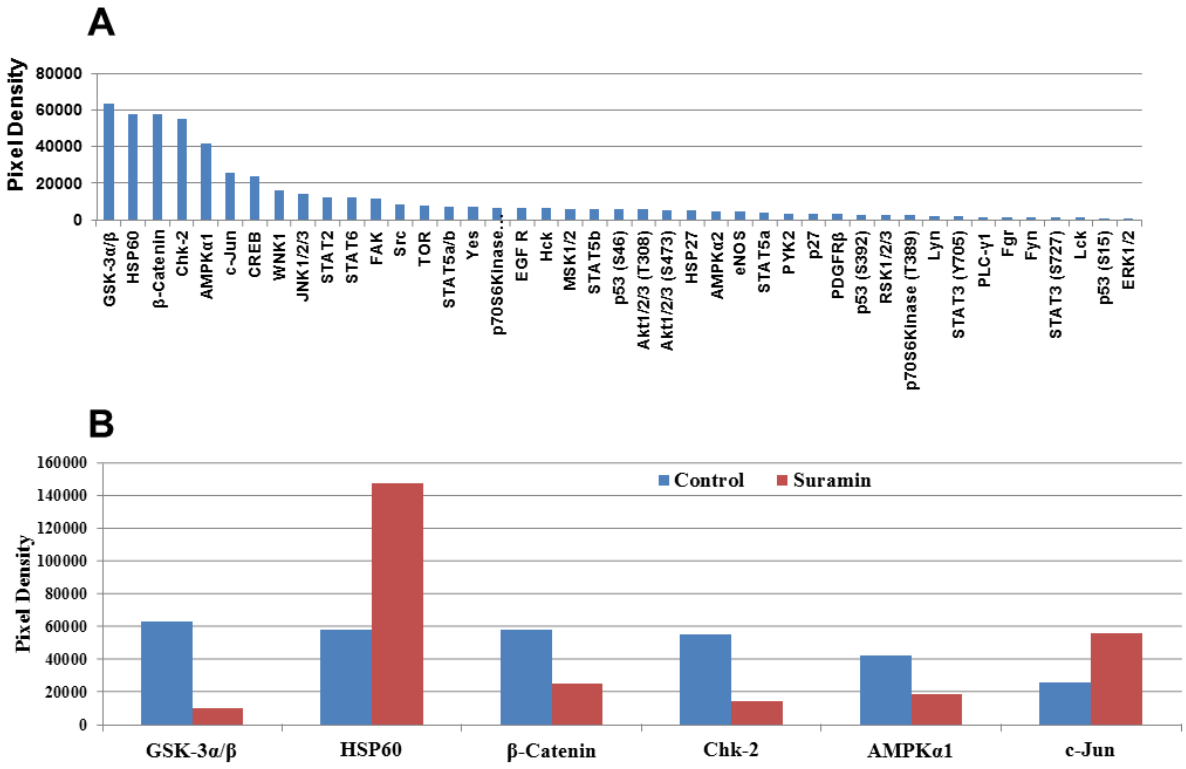

Figure 7: Modification of Phospho-kinase activity in KATO-III cells after treatment with suramin.

6 different phosphorylated proteins were observed in KATO-III treated with suramin (200µM) in comparison to the control. 2 of them were up-regulated (HSP60 and C-Jun) while 4 (GSK-3α/β, β-catenin, Chk-2 and AMPkα-1) were down-regulated.

Table 1

mRNA expression ratio	E-Cadherin	Snail	Slug	Vimentin	α-SMA
Non-adherent/adherent	1.32	0.89	0.8	0.78	0.88
Non-adherent/adherent after grown separately	1.24	0.89	0.9	0.81	0.75

Real-time PCR analysis showed no alteration in gene expression of epithelial-mesenchymal transition (EMT)-related molecules when adherent and non-adherent KATO III cell lines were grown separately for one week. These results so far presented (Figure1-7) provide data that are important in elucidating the goal of the present work.

Discussion

In order to determine whether the high level of HPSE found in SRCA (both tumor and ascitic fluid) is involved in the tumor development and in fibrosis, we analyzed epithelial-mesenchymal transition after addition of an inhibitor of heparanase (suramin) and after culture of cancer cells in culture medium targeted for stem cell differentiation in adipocytes, chondrocytes, osteocytes and neuronal cells.

HPSE is a multitasking protein characterized by enzymatic and non-enzymatic activities. Signaling activity of HPSE is achieved by interacting with transmembrane proteins, modulating the activity of factors such as FGF-2 and TGF-β [624].

HPSE expression was found high in the ascitic samples of the patient with SRCA of stomach as compared to that of patients with non-SRCA of stomach and colic cancer. Furthermore, high HPSE mRNA expression was found in gastric SRCA (KATO-III cell line) as well as in an ovarian cancer cell line (OVCAR-3), human microvascular endothelial (HMEC) as compared to other cancer cell lines such as another ovarian cancer cell (SKOV-3), breast (MDA-MB231 and MCF7), gastric non-SRCA (AGS), intestinal (LS174T), lung (A549), leukemia (K562) and cervical (HELA) cell lines. Active heparanase was also analyzed in the supernatants of in supernatants of cell lines including gastric (KATO-III). SRCA tissue of stomach showed more mRNA expression of HPSE, pro-fibrotic markers (TGF-β1, FGF-2), VEGF-A, mesenchymal markers (Slug, Vimentin) and fibrotic markers (fibronectin, α-SMA and Collagen-I) than those present in the corresponding peritumoral sites.

These findings allow conclude that SRCA are mesenchymal cells expressing high levels of heparanase and fibrotic markers. Our results are compatible with the report of David *et al.* who found HPSE association with intestinal fibrosis *in vivo* [510] and that of Mosala *et al,* who found FGF-2 dependent EMT related fibrosis in diabetic nephropathy [506]. Our results are also in accordance with the findings reported by the two authors cited previously.

FGF-2 was found absent in both forms of KATO-III cells (adherent and non-adherent) and in low amount in tumor tissues of patients with SRCA of stomach compared to higher amount in peri-tumoral areas, suggesting its effect emanating from peri-tumoral sites. The interference of FGF-2 with heparanase [634] as well as

TGF-β[635] and IGF-2 [636] for CD222 activity in the tumor microenvironments may modulate epithelial-mesenchymal interactions [506]. We also observed high mRNA expression of IGF2 in gastric SRCA tumoral tissue than peritumoral tissues (p = 0,004) as well as gastric non-SRCA (p = 0,007). (Results not shown). It also affects TGF-β [637] and FGF-2 [638] related functions and inhibits multilineage differentiation of mesenchymal cells [639] as well as promote tissue fibrosis, cancer expansion and malignancy.

Multi-drug resistance (MDR) remains a great obstacle to effective chemotherapy for SRCA gastric cancer. [640] The present work also indicates, for the first time, that in SRCA tumoral cells, LRP and MDR-1 proteins were expressed higher than other resistance proteins (MRP1-5, BCRP) in tumoral sites of SRCA.

We showed also that KATO-III cells were capable of conversion between two distinct forms (adherent and non-adherent cells), a transition attributed to a reversible adaptive plasticity. We found no alteration in gene expression of epithelial-mesenchymal transition (EMT)-related molecules when adherent and non-adherent cells were grown separately for one week. This finding is consistent with the observation of Jun-Jun She *et al.* who found no difference in tumorigenicity *in vivo* when "*side population*" and "*non-side population*" of KATO-III cells were injected subcutaneously in nude mice [641].
However, we identified major biologically active proteins secreted by non-adherent cells such as Siglec-5, TGF-α and prolactin while the adherent cells secreted TIMP-4, activin-A and CXCL-16. There is evidence that Siglecs-5 is involved in cell-cell interaction and tumor dissemination [642, 643] while TGFα regulates cell proliferation and migration through activation of multiple pathways[644] suggesting that tumor dissemination and cell proliferation are due to the non-adherent KATO III cells.

In addition, we observed spheroid clusters of KATO-III that originated from both adherent and non-adherent cells. Size of these spheroid clusters that originated from non-adherent cells was found to be significantly larger than those that originated from adherent cells. It is well established that Spheroid clusters were found when stem cells from a variety of normal and tumor tissues were isolated[645-647], suggesting that spheroid formation may be a common growth characteristic of SRCA cell line. LRP

and MDR-1 expression by SRCA tumor, an EMT characteristic of this tumor, indicated the chemoresistance of SRCA patients to treatments. We showed that SRCA cells line (KATO-III) present in the CD90 and CD117 positive fractions has a potential to differentiate into adipocytes, chondrocytes, osteocytes and neurocytes confirming their stem cell nature.

In addition, a non-cytotoxic dose of suramin enhances the antitumor effects of several chemotherapeutic agents in a number of tumor cell lines [648] and several animal models [649]. A growing body of evidence has shown that suramin is the inhibitor of an enzyme, HPSE [539]. After finding heparanase mRNA and protein in KATO-III cells, we treated these cells with suramin and observed increased phosphorylation of HSP60 and c-Jun while GSK-3α/β, β-catenin, Chk-2 and AMPkα-1 decreased. These results suggest the crucial role of heparanase in KATO III cell homeostasis. GSK-3 has been associated with tumor progression by stabilizing components of the β-catenin complex [650]. Chk-2 as a kinase is involved in cell differentiation [651] and may influence SRCA differentiation and cell fibrosis while activation of AMPkα-1 acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP for cell homeostasis [652]. C-jun phosphorylation is required for maintaining sufficient cyclin D1 kinase activity and for allowing cell cycle progression and apoptosis [653, 654].

Our interest was to focus on the regulation of epithelial mesenchymal transition and fibrosis by altering the expression of EMT markers as well as TGF-β and collagen-I expression respectively using suramin. This was investigated in KATO-III cells in which we showed significant inhibitory effect of suramin on heparanase and collagen-I related fibrosis. Our findings demonstrate that suramin exerts a considerable degree of inhibition on EMT and proliferation in KATO-III cells. Similarly, suramin also induced a G0/G1 cell cycle block of up to 39% and inhibited S phase up to 98% of the cell population. These findings are similar to those reported by HuaPing Li *et al.* who found inhibition of cell proliferation by suramin in ovarian and cervical cancer by down regulating heparanase expression [655]. These results are in good correlation with our results showing that heparanase regulated epithelial mesenchymal transition is inhibited by suramin, an heparanase inhibitor.

Conclusion

In the scenario of a progressive gastric fibrosis like signet ring cell adenocarcinoma of stomach, HPSE (which is increased in gastric microenvironment) is responsible for pro-fibrotic factor dependent EMT leading to cell malignancy and fibrosis. The expression level of LRP and MDR-1 was found higher in signet ring cell adenocarcinoma which contributes to the chemoresistance observed in this malignancy. Suramin has been shown to be effective in the prevention and treatment of the EMT related fibrosis markers. HPSE could therefore be an interesting pharmacological target for the treatment of gastric signet ring cell adenocarcinoma.

Disclosures:

The authors have no financial conflicts of interest.

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Complementary document.

Sequence of primers for RT and real-time PCR

Gene	Primers (5`→3`)	Product length (bp)
Hepranase	AGACGGCTAAGATGCTGAAGAG	208
	TCTCCTAACCAGACCTTCTTGC	
FGF-2	CTGGCTATGAAGGAAGATGGA	149
	TGCCCAGTTCGTTTCAGTG	
TGF-β1	CAGAAATACAGCAACAATTCCTGG	186
	TTGCAGTGTGTTATCCCTGCTGTC	
VEGF-A	CCCACTGAGGAGTCCAACAT	173
	AAATGCTTTCTCCGCTCTGA	
E-Cadherin	TGGACAGGGAGGATTTTGAG	190
	ACCTGAGGCTTTGGATTCCT	
Snail	CCAATCGGAAGCCTAACTACAG	155
	GACAGAGTCCCAGATGAGCATT	
Slug	GCATTTCTTCACTCCGAAGC	151
	TGAATTCCATGCTCTTGCAG	
Vimentin	GAGAACTTTGCCGTTGAAGC	163
	GCTTCCTGTAGGTGGCAATC	
α-SMA	TTCAATGTCCCAGCCATGTA	222
	GAAGGAATAGCCACGCTCAG	
Collagen-I	CCTGGATGCCATCAAAGTCT	153
	AATCCATCGGTCATGCTCTC	
Fibronectin	CCGAGGGACCTGGAAGTT	151
	ACTTGCTCCCAGGCACAG	
MRP-1	AGGTGGACCTGTTTCGTGAC	181
	CCTGTGATCCACCAGAAGGT	
MRP-2	GACCAACATTGTGGCTGTTG	163
	GAGGACCAGATCCAGCTCAG	
MRP-3	GGGCGTCTATGCTGCTTTAG	188

	CCTTGGAGAAGCAGTTCAGG	
MRP-4	AGAGCTGGTGCTCACTGGAT	154
	CGGTTACATTTCCTCCTCCA	
MRP-5	CCTTTTCACTCCCTCCATCA	185
	ACAGGTCTTGGAGCTGGAGA	
BCRP	CACCTTATTGGCCTCAGGAA	200
	CCTGCTTGGAAGGCTCTATG	
MDR-1	TGCCACCACGATAGCTGA	172
	CTGCTTCTGCCCACCACT	
LRP	GTGGAGGTCGTGGAGATCAT	186
	CCAAATCCAGAACCTCCTCA	
β-ACTIN	AGAGCTACGAGCTGCCTGAC	184
	AGCACTGTGTTGGCGTACAG	

References

1. Otsuji E, Kuriu Y, Okamoto K, et al. Outcome of surgical treatment for patients with scirrhous carcinoma of the stomach. *The American journal of surgery* 2004; **188**(3): 327-32.
2. Yonemura Y, Endou Y, Sasaki T, et al. Surgical treatment for peritoneal carcinomatosis from gastric cancer. *European Journal of Surgical Oncology (EJSO)* 2010; **36**(12): 1131-8.
3. Huh HJ, Park C-J, Jang S, et al. Prognostic significance of multidrug resistance gene 1 (MDR1), multidrug resistance-related protein (MRP) and lung resistance protein (LRP) mRNA expression in acute leukemia. *Journal of Korean medical science* 2006; **21**(2): 253-8.
4. Vtorushin S, Khristenko K, Zavyalova M, et al. The phenomenon of multi-drug resistance in the treatment of malignant tumors. *Exp Oncol* 2014; **36**(3): 144-56.
5. Sui H, Zhu L, Deng W, Li Q. Epithelial-mesenchymal transition and drug resistance: role, molecular mechanisms, and therapeutic strategies. *Oncology research and treatment* 2014; **37**(10): 584-9.
6. Nieto MA. The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annual review of cell and developmental biology* 2011; **27**: 347-76.
7. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**(5): 646-74.
8. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**(5): 871-90.
9. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *The Journal of cell biology* 2006; **172**(7): 973-81.
10. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *Journal of Clinical Investigation* 2003; **112**(12): 1776.
11. Yashiro M, Matsuoka T. Scirrhous gastric cancer: a critical review. *OA Cancer* 2014; **2**(4).
12. Masola V, Zaza G, Secchi MF, Gambaro G, Lupo A, Onisto M. Heparanase is a key player in renal fibrosis by regulating TGF-β expression and activity. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 2014; **1843**(9): 2122-8.
13. Buø L, Karlsrud T, Dyrhaug G, et al. The fibrinolytic system in human ascites. *Scandinavian journal of gastroenterology* 1995; **30**(11): 1101-7.
14. Secchi MF, Masola V, Zaza G, Lupo A, Gambaro G, Onisto M. Recent data concerning heparanase: focus on fibrosis, inflammation and cancer. *Biomolecular concepts* 2015; **6**(5-6): 415-21.
15. Wood RJ, Hulett MD. Cell surface-expressed cation-independent mannose 6-phosphate receptor (CD222) binds enzymatically active heparanase independently of mannose 6-phosphate to promote extracellular matrix degradation. *Journal of Biological Chemistry* 2008; **283**(7): 4165-76.
16. Nakajima M, Irimura T, Nicolson GL. Heparanases and tumor metastasis. *Journal of cellular biochemistry* 1988; **36**(2): 157-67.
17. Vlodavsky I, Miao H-Q, Medalion B, Danagher P, Ron D. Involvement of heparan sulfate and related molecules in sequestration and growth promoting

activity of fibroblast growth factor. *Cancer and Metastasis Reviews* 1996; **15**(2): 177-86.
18. Cohen T, Gitay-Goren H, Sharon R, et al. VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *Journal of Biological Chemistry* 1995; **270**(19): 11322-6.
19. Vlodavsky I, Bar-Shavit R, Ishar-Michael R, Bashkin P, Fuks Z. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? *Trends in biochemical sciences* 1991; **16**: 268-71.
20. Goldshmidt O, Zcharia E, Cohen M, et al. Heparanase mediates cell adhesion independent of its enzymatic activity. *The FASEB Journal* 2003; **17**(9): 1015-25.
21. Fux L, Ilan N, Sanderson RD, Vlodavsky I. Heparanase: busy at the cell surface. *Trends in biochemical sciences* 2009; **34**(10): 511-9.
22. Masola V, Gambaro G, Tibaldi E, et al. Heparanase and syndecan-1 interplay orchestrates fibroblast growth factor-2-induced epithelial-mesenchymal transition in renal tubular cells. *Journal of Biological Chemistry* 2012; **287**(2): 1478-88.
23. Koliopanos A, Friess H, Kleeff J, et al. Heparanase expression in primary and metastatic pancreatic cancer. *Cancer research* 2001; **61**(12): 4655-9.
24. HIROKIKUNIYASU YC, KUBOZOE T, TAKAHASHI T. Co-expression of CD44v3 and heparanase is correlated with metastasis of human colon cancer. *International journal of molecular medicine* 2002; **10**: 333-7.
25. Kim AW, Xu X, Hollinger EF, Gattuso P, Godellas CV, Prinz RA. Human heparanase-1 gene expression in pancreatic adenocarcinoma. *Journal of gastrointestinal surgery* 2002; **6**(2): 167-72.
26. Rohloff J, Zinke J, Schoppmeyer K, et al. Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma. *British journal of cancer* 2002; **86**(8): 1270-5.
27. Uno F, Fujiwara T, Takata Y, et al. Antisense-mediated suppression of human heparanase gene expression inhibits pleural dissemination of human cancer cells. *Cancer research* 2001; **61**(21): 7855-60.
28. Davids JS, Carothers AM, Damas BC, Bertagnolli MM. Chronic cyclooxygenase-2 inhibition promotes myofibroblast-associated intestinal fibrosis. *Cancer Prevention Research* 2010; **3**(3): 348-58.
29. Marchetti D, Reiland J, Kempf D, Roy M, Denkins Y. FGF2 binding, signaling and angiogenesis are modulated by heparanase in metastatic melanoma cells. *Melanoma Research* 2006; **16**: S67.
30. Zhang J, Wong MG, Wong M, et al. A cationic-independent mannose 6-phosphate receptor inhibitor (PXS64) ameliorates kidney fibrosis by inhibiting activation of transforming growth factor-β1. *PloS one* 2015; **10**(2): e0116888.
31. Leksa V, Pfisterer K, Ondrovičová G, et al. Dissecting mannose 6-phosphate-insulin-like growth factor 2 receptor complexes that control activation and uptake of plasminogen in cells. *Journal of Biological Chemistry* 2012; **287**(27): 22450-62.
32. Dennis PA, Rifkin DB. Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proceedings of the National Academy of Sciences* 1991; **88**(2): 580-4.

33. Ishai-Michaeli R, Eldor A, Vlodavsky I. Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell regulation* 1990; **1**(11): 833-42.
34. Lai WT, Krishnappa V, Phinney DG. Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels. *Stem cells* 2011; **29**(7): 1102-11.
35. Wu J, Qin H, Li T, et al. Characterization of site-specific glycosylation of secreted proteins associated with multi-drug resistance of gastric cancer. *Oncotarget* 2016.
36. She J-J, Zhang P-G, Wang X, Che X-M, Wang Z-M. Side population cells isolated from KATO Ⅲ human gastric cancer cell line have cancer stem cell-like characteristics. *World J Gastroenterol* 2012; **18**(33): 4610-7.
37. Crocker PR. Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell–cell interactions and signalling. *Current opinion in structural biology* 2002; **12**(5): 609-15.
38. Fu-Tong L, Rabinovich GA. Galectins as modulators of tumour progression. *Nature reviews Cancer* 2005; **5**(1): 29.
39. Wang C, Lv X, Jiang C, et al. Transforming growth factor alpha (TGFα) regulates granulosa cell tumor (GCT) cell proliferation and migration through activation of multiple pathways. *PLoS One* 2012; **7**(11): e48299.
40. Dontu G, Abdallah WM, Foley JM, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development* 2003; **17**(10): 1253-70.
41. Toma JG, Akhavan M, Fernandes KJ, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nature cell biology* 2001; **3**(9): 778-84.
42. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer research* 2003; **63**(18): 5821-8.
43. Song S, Wientjes MG, Gan Y, Au JL-S. Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs. *Proceedings of the National Academy of Sciences* 2000; **97**(15): 8658-63.
44. Blum JL, Dees EC, Chacko A, et al. Phase II trial of capecitabine and weekly paclitaxel as first-line therapy for metastatic breast cancer. *Journal of clinical oncology* 2006; **24**(27): 4384-90.
45. Marchetti D, Reiland J, Erwin B, Roy M. Inhibition of heparanase activity and heparanase-induced angiogenesis by suramin analogues. *International journal of cancer* 2003; **104**(2): 167-74.
46. McCubrey JA, Steelman LS, Bertrand FE, et al. GSK-3 as potential target for therapeutic intervention in cancer. *Oncotarget* 2014; **5**(10): 2881.
47. Takagaki K, Katsuma S, Kaminishi Y, et al. Role of Chk1 and Chk2 in Ara-C-induced differentiation of human leukemia K562 cells. *Genes to Cells* 2005; **10**(2): 97-106.
48. Cantó C, Gerhart-Hines Z, Feige JN, et al. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* 2009; **458**(7241): 1056.
49. Wisdom R, Johnson RS, Moore C. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *The EMBO journal* 1999; **18**(1): 188-97.

50. Bossy-Wetzel E, Bakiri L, Yaniv M. Induction of apoptosis by the transcription factor c-Jun. *The EMBO journal* 1997; **16**(7): 1695-709.
51. Li H, Li H, Qu H, et al. Suramin inhibits cell proliferation in ovarian and cervical cancer by downregulating heparanase expression. *Cancer cell international* 2015; **15**(1): 52.

Article No 3

Fibrin deposit on the peritoneal surface serve as a niche for cancer expansion in carcinomatosis patients

Ovarian carcinomatosis microenvironments induce epithelial-mesenchymal-transition and up-regulate protease-procoagulant activity in mesothelial cells

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Running title: Involvement of mesothelial cells in carcinomatosis expansion

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The highlights of the present work:

1-Changes in mesothelial cell morphology by tumor microenvironment induced neprilysin and proteolytic enzymes and aid their detachment from peritoneal surface.

2- Modification of peritoneal pro-coagulant activity for fibrin network generation.

3-The niche of cancer cell clusters on the fibrin deposits of peritoneal surface may lead to carcinomatosis expansion.

Abstract:

The expansion of carcinomatosis in the peritoneal cavity is poorly documented. Here we studied the effect of microenvironments on modified mesothelial layer hemostasis and fibrin deposition for cancer cell adhesion and dissemination. Peritoneal membrane obtained from the digestive surgery department. Immunohistochemistry was performed using antibodies: E-cadherin, cytokeratin, CD-31, Ki67, WT-1 and D2-40. Scanning (SEM) and transmission electron microscopy of peritoneal surface and cancer cell clusters from cancer patients was done. Ascites and its impact on mesothelial cells were assessed by cytokine array. Neprilysin, matrix metalloprotease, epithelial mesenchymal transition (EMT) related molecules (E-cadherin, Snail, Slug, Twist, Vimentin, N-cadherin and Fibronectin), tissues factor (TF), endothelial protein C receptors (EPCR) were quantified by q-PCR. Migration ability was assessed by scratch assay. Cell viability and neprilysin activity were analyzed by bioluminescence. Cancer cells-fibrin interaction was investigated by SEM and microcinematography. Our results demonstrated that, carcinomatosis microenvironments induce mesothelial cells to: 1) change their morphology, 2) up-regulate EMT markers, 3) modify their behavior by upregulation of neprilysin, MMP2, TF and cytokines secretions, 4) detach from peritoneum, and 5) up-regulate interleukin-6, and 8, hepatocyte growth factor and granulocyte chemotactic protein-2 mRNA expression. Ascites cancer cell clusters were found to be associated with fibrin and fibrin deposits on the peritoneal surface. Kinetics of cancer cell-fibrin interaction *in vitro* showed that fiber filaments expansion from clots inhibited cancer cell adhesion on peritoneal surface. In conclusion, for first time, we demonstrate the role of fibrin net generated on the peritoneal surfaces in cancer cell dissemination and carcinomatosis expansion.

Keywords: Peritoneal carcinomatosis; Mesothelial cells; Fibrin network; Tissues factor; Neprilysin.

Introduction:

Ovarian cancers often accompanied by ascites, is one of the gynecological malignancies characterized by metastatic potential to disseminate tumor cells inside the peritoneum [656]. Peritoneal dissemination results in poor prognosis and is a leading cause of death in patients.

The tumor sheds cells into the peritoneal cavity which implant on a membrane (mesothelium) and cover the peritoneal surfaces. Complex bidirectional interactions between metastatic cancer cells and peritoneal environment seem to be crucial for colonization on the peritoneum wall. Epithelial mesenchymal transition (EMT) in mesothelial cells has been reported to play an important role in the processes of attaching to and invading through the peritoneal membrane [657].

Mesothelial monolayer lines the peritoneal cavity and its associated organs and are the major site of secondary tumor development[658]. Several hypotheses such as adhesion of cancer cell via mesothelial cells or mesothelial basal membrane were proposed[659] and the role of VCAM-1 [660], α3β1 integrin [661] as well as MMP [662], TGFβ [663], EGF [664], HGF [665] and VEGF-A and C were investigated [666].

Mesothelial cells are flat cells producing a small amount of lubricating fluid inside the abdomen with a dynamic cellular membrane. The primary function of this layer is to provide a slippery, non-adhesive and protective surface [667]. However, mesothelial cell layers play other essential roles involving the transport of fluids and cells through the serous cavities, antigen presentation, inflammation, coagulation, fibrinolysis and tumor cell adhesion in carcinomatosis [156]. Mesothelial cell layers are the first target in different peritoneal diseases such as infection or carcinomatosis [668].

Here we studied the expression of procoagulant and proteolytic enzymes within tumor microenvironment to modify peritoneal surfaces during carcinomatosis expansion.

Materials and Methods

Cell lines

Normal human adult mesothelial cells were purchased from Zen Bio, Inc. (Research Triangle Park, NC, USA) and CT-26 (colon cancer) from American Type Culture Collection (ATCC, Manassas, VA).Both cells (mesothelial cells and CT26) were maintained in mesothelial cell growth medium (Zen-Bio, Inc.) and DMEM (Gibco, Saint Aubin, France) respectively. The cellular environment was maintained at 50 mL/L CO_2 and 37℃.

Patients:

Peritoneal membranes (ovarian cancer patient) and six freshly-isolated ascites fluids (ovarian n=2, gastric n=2 and colic n=2 cancer patients) were obtained from General and Digestive Tract Surgery Department at Lariboisière Hospital in Paris (France). Informed consent was obtained from each patient prior to surgery. Cells ($2x10^5$/200 µlit) from peritoneal liquid (n=3) were pelleted by a short spin at 3000 rpm for 10 minutes at 20^0C. Ascites fluids obtained from cancer patient (n=4) were used after centrifugation at 1200 rpm for 5 min and preserved at -80°C.

Fluorometric assays

A substrate based activity assay kit (AnaspecSensoLyte®, Belgium) that determines neprilysin activity was utilized according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of mesothelial cells grown in medium with or without 25% ascites for 6 days were used. Aliquots from each sample were incubated in the presence of the neprilysin substrate solutions for 60 min. The fluorescent product was measured in a spectrophotofluorometer (GloMax®-Multi Detection System, France) with excitation at 490 nm and emission at 520 nm.

Immunohistochemistry

Invasive and non-invasive peritoneum as well as tumor tissue samples were obtained from patients and used for this study. For anatomo-pathological analysis, the samples were dissected, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. The slides (4 micron) were prepared and colored by hematein-eosin-safran according to classical methods in the anatomo-pathogical laboratories. Similarly, slides were also prepared using cytospin for ascitic cells of ovarian cancer. In parallel several slides were stained with antibodies coupled peroxidase using Benchmark Ultra apparatus

(Roche Ventana, Tucson, Arizona, USA) according to the protocol of their manufacturers. Antibodies used for immunohistochemistry were as follows: antiE-cadherin (1/50), Ki67 (1/50), CD-31 (1/20), D2-40 (not diluted)from Glostrup USA and cytokeratin (1/50), WT-1 (1/50) from Carpinteria-Californie USA. The pictures were taken by Leitz (Diaplan) microscopy with Nikon Cool pix 995 apparatus (Japan).

Scanning Electron Microscopy

Peritoneal membranes were fixed using 4% PFA for 24hr and washed with PBS (1x) buffer three times each for 5 min. After this, 2% glutaraldehyde was added on the membrane for 20 minutes and washed with PBS (1x) buffer three times each for 5 min. After a final wash in distilled water, the samples were dehydrated in increasing concentrations of ethanol. Samples were sputter-coated with gold after drying. They were observed with a S260 CAMBRIDGE scanning electron equipped with a LaB6 filament operating at 15kV and images were captured with the software "Orion" from (NCH Software).

Transmission Electron Microscopy

Primary ovarian cancer cells with fibrosis were detected using transmission electron microscopy. Briefly, primary cells, after recovering from ascites, were grown in DMEM medium. Cells (100 millions) were harvested, pelleted, fixed in 4% PFA for 15 minutes. The samples were then rinsed with PBS (1X). Ultrathin sections (50–70 nm) were cut using a Leica RM2235 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and observed with a transmission electron microscope (Hitachi H-800; Hitachi, Tokyo, Japan).

Microcinematography

To document the kinetics of CT26 cells in the presence of fibrin, time-lapse microcinematography was done. Briefly, 100µl pool of human plasma with 2 units of thrombin and 30µl of 0.025M $CaCl_2$ (Diagnostic STAGO, Parsippany, USA) was prepared in a 6-well plate. After 30-45 minutes, CT26 cells (5×10^5/well) were seeded on thrombin clot in medium. The plate was placed in the stagetop environmental chamber. A specific area was focused where there was a maximum chance to observe the interaction between cancer cells and thrombin clot. Microcinematography was

performed to acquire images every 2 minutes for 24 and 48hrs to study the migration behavior of CT26 in a temperature controlled room of 37 ^{0}C in a humidified atmosphere (>80%) containing 5% CO2 by use of an EVOS® FL Auto Imaging System (Life Technologies™ Waltham, USA). The distance travelled by cancer cells was calculated in micrometer while average speed in micrometer/min was calculated by total distance travelled divided by the time required to travel that distance.

Wound healing assay

Mesothelial cells were grown (80% confluency) in 12 well plate with or without 25% ovarian ascites for 6h, 12h, 24h or 36h. The migration ability of mesothelial cells was evaluated by means of a scratch assay. A denuded area was generated on quiescent cell monolayers of mesothelial cells by scratching with a sterile pipette tip. The monolayer was washed twice with PBS (1x) and then incubated in medium having no FBS. The cells were photographed at different time points. The width of the scratch was measured at three different places on the photograph to obtain a mean value after time different intervals. The closing rate of the wound (µm/hr) was calculated as the slope of the line obtained after plotting a graph; width versus time interval.

RNA isolation, RT and real-time PCR

Total RNA in the cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. RNA samples were transcribed to cDNA in a 20 µl volume, using the QuantiTect reverse transcription kit (Qiagen).

The thermal cycling comprised of the real time PCR following conditions: 95 ^{0}C for 5min, followed by 40 cycles (denaturation for 15s at 95 ^{0}C, annealing for 20s at 60 ^{0}C) and extension for 20s at 72 ^{0}C). The primers sequences and PCR product size for the target and reference gene are listed in Complementary result 1.
mRNA expression levels of different markers were detected by real-time PCR with ß-actin as internal reference, using Mesa Blue qPCR Master Mix Plus for SYBR assay (Eurogentec) on the Mastercycler Realplex2 (Eppendorf).

Relative quantitation was calculated using the comparative threshold cycle (C_T) method with realplex software. Mean C_T of triplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (ß-actin) genes. The difference between the ΔC_T of the control experiment (mesothelial cells) and the

ΔC_T of each sample were calculated to give $\Delta\Delta C_T$. Fold increase in mRNA was calculated by $2^{-\Delta\Delta CT}$.

The PCR products of cell lines and tissue samples, after real-time PCR, were electrophoresed by E-Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific Inc, Waltham, USA).

Cytokine array

We examined the supernatant of mesothelial cells grown in 25% ovarian ascites using a protein cytokine array (RayBio ® Human Cytokine Antibody, Norcross, GA). This technique is based on the principle of sandwich immunoassay. It comprises essentially of screening, in duplicate, 174 different membrane-coupled anti-cytokines along with appropriate controls. Mesothelial cells ($0.25x10^6$ cells per ml) were incubated in RPMI medium having no fetal calf serum with or within 25% ovarian ascites at 37˚C in a humidified atmosphere of 5% CO_2 for 24h. Supernatants containing cytokines were retrieved and the cytokines were allowed to couple with their specific antibodies previously immobilized on membranes. Membranes were saturated for 2h at room temperature with bovine serum albumin (BSA). Incubation of array membranes with supernatants was carried out overnight at 4˚C using corresponding antibodies. After several successive washes, membranes were incubated in the presence of a mixture of antibodies and anti-cytokines biotinylated at 4˚C overnight. Streptavidin, coupled with HRP, was added on the membranes for 2 h at room temperature. The presence of antibody coupled proteins was revealed by applying ECL (enhanced chemiluminescence) to the membranes, according to the recommendations of the manufacturer. Membranes were then exposed to photosensitive film (Kodak, X-OMAT, AR, USA).

The intensity of chemiluminescence captured on the photo-sensitive film was measured and recorded. After subtracting the background noise, the results were expressed as a ratio of chemiluminescence intensity of experimental versus control spots.

Cell viability assay

Cell viability was assayed by Real Time-Glo™ MT Cell Viability Assay. In brief, cells (5×10^2/well) were seeded in 96-well plates, following 24 h later by treatment with 25% gastric, colic or ovarian ascites for 48 h. Bioluminescence was measured with spectrofluorometer SAFAS Xenius XC. Cell viability was expressed as the percentage of absorbance of the ascites-treated cells relative to that of the vehicle-treated cells. Each condition was done in triplicate. The experiment is representative of three independent lots.

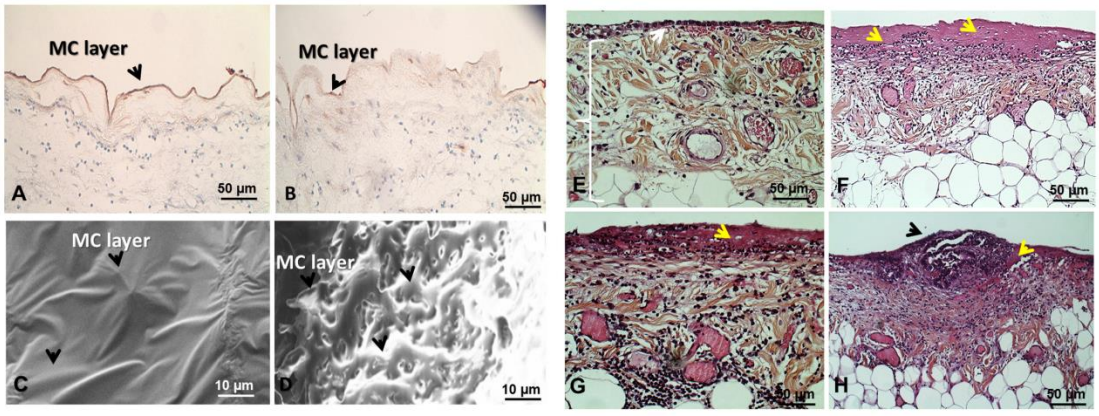
Results:

1- The change of mesothelial cell layer morphology and fibrin deposition on the peritoneum wall

As presented in figure 1, mesothelial cell layer in non-invasive region of peritoneum form a continuous line when the cells of the peritoneal sample were stained by E-cadherin (Figure 1A). In invasive regions, the mesothelial cell layer is perturbed in some areas of the peritoneal cavity and form a discontinue line under the same conditions (Figure 1B). Figure 1C presents the scanning electron microscopy (SEM) analysis of non carcinomatosis peritoneal cavity. The mesothelial cells (arrow) with the plicate layer show the normal cell-cell interaction forming a homogenous layer. Figure 1D shows SEM analysis of the invasive peritoneum in which detachment of mesothelial cells from sub mesothelial cell layer was noticed.

Fibrin deposits on the mesothelial cell layer of peritoneal carcinomatosis were analyzed by classical anatomo-pathological studies. As presented in figure 1E, non-invasive peritoneum wall in carcinomatosis patients were analyzed by photonic microscopy. As shown in figure 1F-H, several regions with fibrin deposition in the inter-mesothelial cells space were observed. In some regions, cancer nodules were observed on the fibrin net (Figure 1H). The presence of fibrin on the peritoneal wall may be a consequence of procoagulant activity via mesothelial tissues factor as well as sub mesothelial collagen in these regions.

SEM analysis of non-invasive peritoneum shows an unchanging and unbroken surface covered by mesothelial cells (Figure 1I). The surface of the mesothelial layer is intact and the cells are naturally arranged. In contrast, in invasive regions, the mesothelial cells were deformed and fiber nets were deposited within the inter-mesothelial cell space (Figure 1J and 1K). The interaction of cancer cells with fibrin deposit is presented in figure 1L.



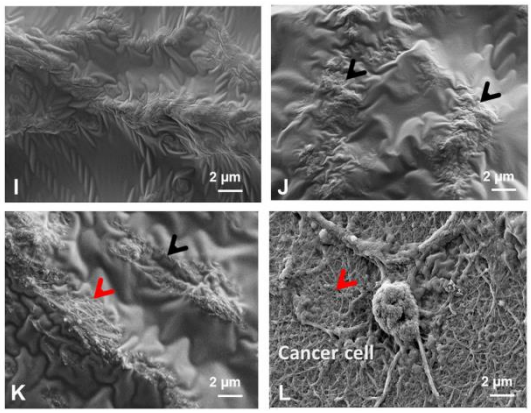


Figure 1. Mesothelial cell layer in non-invasive and invasive region of peritoneum and clusters of malignant cells in ascites:

Invasive and non-invasive region of peritoneum by immunohistochemistry of E-cadherin (A and B) and scanning electron microscopy (C and B). Mesothelial cell (MC) shown by black arrow. Eosin hematoxylin of non-invasive peritoneum was presented in E and mesothelial layer identified by white arrow. Fibrin deposit and cancer nodule on the peritoneal surface presented with yellow and black arrow respectively (F, G and H). Scanning electron microscopy analysis of non-invasive (I) and invasive peritoneum (J, k and L) demonstrated the intermesothelial surface (black arrow). The presence of fibrin network identified on the inter mesothelial (basement membrane) space (red arrow, K and L) and the cancer cell interaction with fibrin network (L).

2- <u>Fibrin deposit on the peritoneum wall associated with angiogenesis and lymphangiogenesis:</u>

Figure 2A-D presents the non-invasive region of peritoneum. The mesothelial cells were stained by anti–cytokeratin antibody that reacts only with epithelial cells (Figure 2A). The blood vessels were stained by anti CD-31 antibody. CD31 was present on the platelets, monocytes, granulocytes, B lymphocytes and intracellular endothelial junction (Figure 2B).The lymphatic wall was stained by anti D2-40 antibody (Figure 2C). This antibody also reacts with mesothelial cells. Figure 2D presents all the cells that are in division cycle stained using Ki67 antibody.

In the invasive regions, the presence of fibrin deposit was associated with high expression of CD31 (Figure 2E) and D2-40 proteins (Figure 2F). As presented in figure 2G, the proliferative cells are present in fibrin deposit as well as peritoneal tissues. The number of blood vessels and lymphatic vessels in non-invasive as well as

invasive regions of peritoneum is presented in figure 2H. Interestingly, the blood vessels in fibrin deposit zone contain more mononuclear cells compared with other zones reflecting previous inflammatory events.

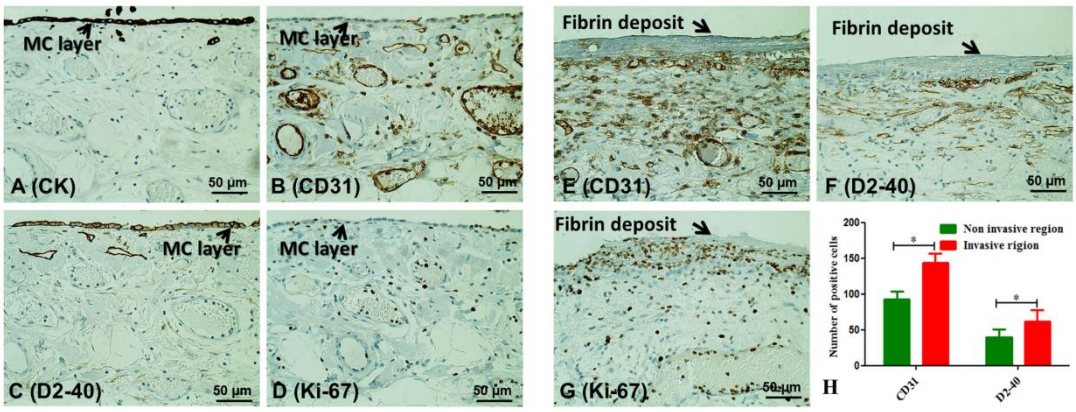


Figure 2. Immunohistochemistry of non-invasive and invasive region of peritoneum:

Immunohistochemical detection of cytokeratin (mesothelial layer), CD31 (endothelial cells of blood vessels), D2-40 (endothelial cells of lymphatic vessels that cross react with mesothelial cells) and Ki-67 (proliferative marker) in non-invasive region (A, B, C and D) and of CD31, D2-40 and Ki-67 in invasive region (E, F and G) of peritoneum were presented. The fibrin deposit identified using black arrows in I, F and G. Statistical data for the number of CD31 and D2-40 stained cells in non-invasive and invasive regions is presented (H). *P<0.05

3- Peritoneal fluid from ovarian carcinomatosis modified mesothelial cell behavior *in vitro*.

For analysis of the impact of ovarian peritoneal fluid on the mesothelial cells, we tested the effect of medium with 25% of ovarian ascites on cell shapes, cell proliferation and cell migration *in vitro*. When mesothelial cells were incubated with ascites, they acquired a fusiform shape. Figure 3 presents mesothelial cell shapes *in vitro* before (3A) and after (3B) incubation with ascites. The numerical results are presented in figure 3C (*p=0.01*). The presence of ascites in culture medium increases significantly mesothelial cell proliferation (*p=0.001*), wound healing and migration (*p=0.01*) in a time dependent manner (Figure 3D-F). These results are in favor of the influence of ascites on the mesothelial cell layer as observed microscopy.

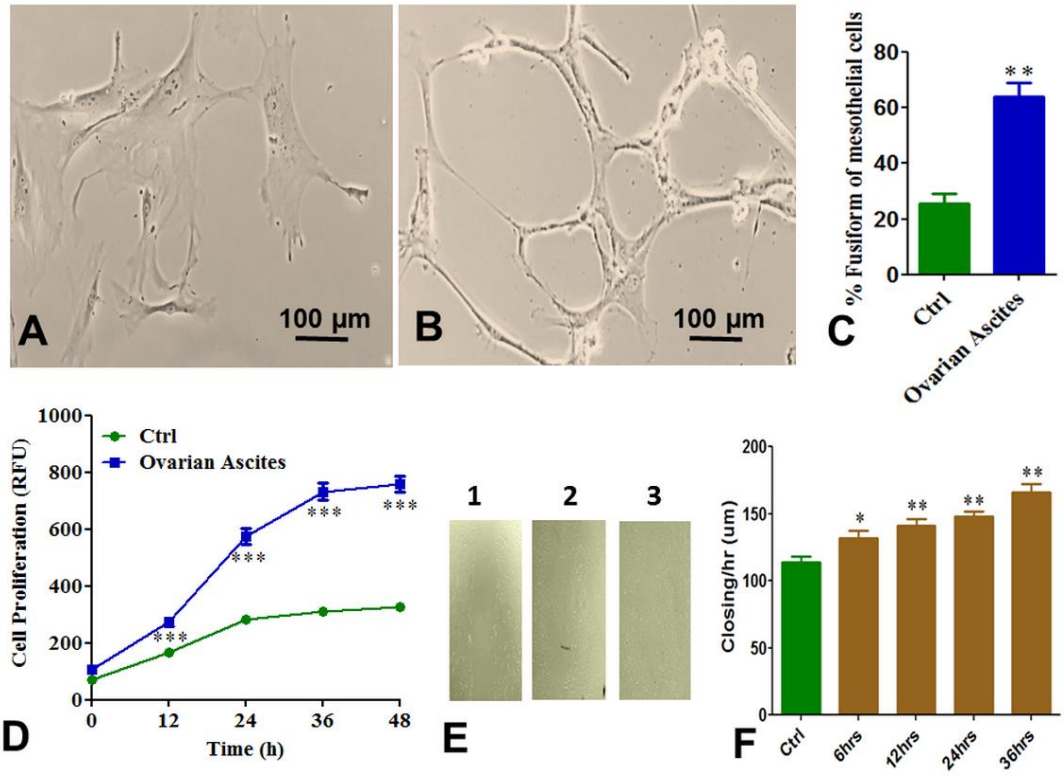


Figure 3. Ovarian ascites induces modification in cell shape, proliferation and migration of mesothelial cells:

Morphology of mesothelial cells before (A) and after (B) treatment with ovarian ascites. % age fusiform of mesothelial cells after treatment of ovarian ascites (C). Mesothelial cells proliferate (D) and migrate (F) rapidly in the presence of ovarian ascites. Shown is a photomicrograph of representative fields of mesothelial cell migration in 25% ovarian ascites (E). The results are expressed as mean ± SEM of six independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the control.

4- <u>Ovarian ascites contain biologically active proteins and modify cytokine secretion by mesothelial cells *in vitro*:</u>

We tested several peritoneal fluids from ovarian carcinomatosis by cytokine array analysis. The pattern of cytokines was detected in two ovarian cancer patients and is presented in complementary result 3. The results indicate the presence of several groups of cytokines in each ascites involved in cancer cell growth and progression, angiogenic and lymphangiogenic stimulation, mesothelial cell activation, immune cell

activation/inhibition, cell adherence, membrane permeabilisation and procoagulant activity. When mesothelial cells were incubated in 25% ascites for 24 h *in vitro* and then in medium without serum and other biological additives (conditioned medium), the profile of cytokines pattern was changed. Mesothelial cells secrete a high amount of IL-6, MCP-1, GRO, ANG, OPG, IL-8 and TIMP-1 in treated conditions. The presence of ovarian ascites in culture medium can modify and increase cytokines as presented in figure 4A. Among up-regulated proteins, the effect of IL-6, IL-8, HGF and GCP2 were tested separately with conditioned medium on mesothelial cells *in vitro*. The levels of mRNA folds for metalloprotease, procoagulant and pro-anticoagulant are presented in the complementary result 2. These results indicate the upregulation of metalloprotease MMPs and neprilysin as well as tissues factor and fibronectin via an autocrine pathway (Figure 4B-E).

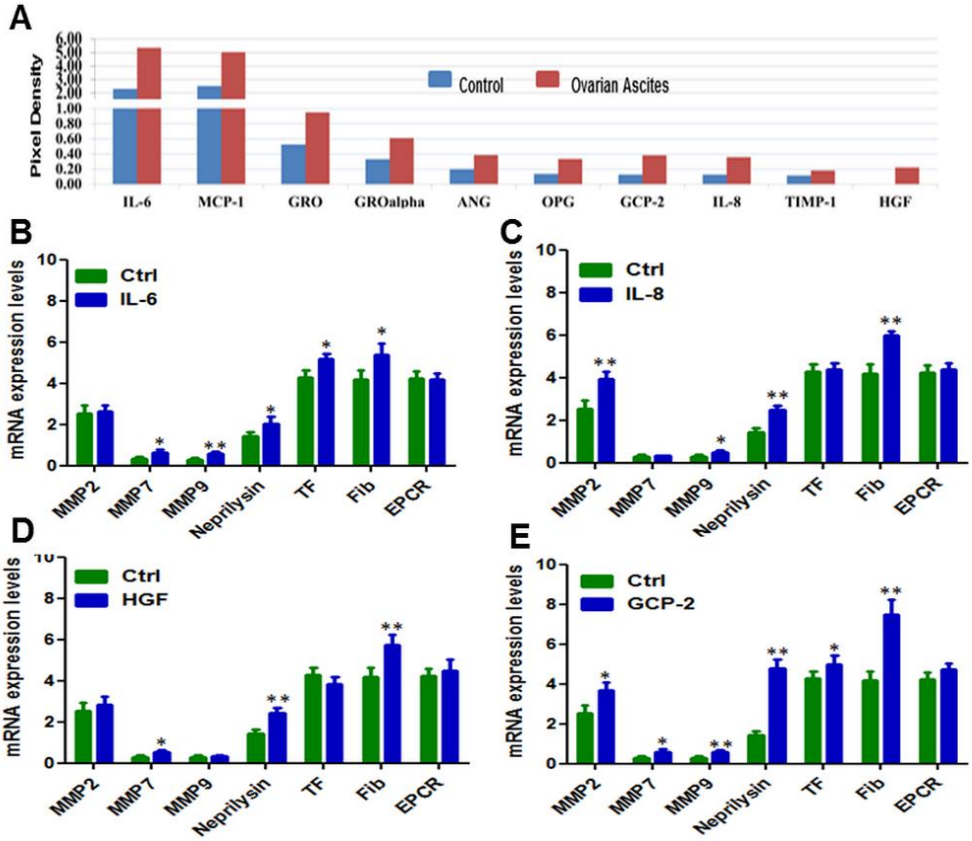


Figure 4. Relative expression of cytokines and mRNA of metalloproteinases, procoagulant and pro-anticoagulants in mesothelial cells after treatment with ovarian ascites:

Histogram shows the relative expression of cytokines (IL-6, MCP-1, GRO, GRO-α, ANG, OPG, GCP-2, IL-8, TIMP-1, HGF) in mesothelial cells after treatment with ovarian ascites **(A).** mRNA of metalloproteinases (MMP-2, MMP-7, MMP-9 and

CD10), procoagulant (TF) and pro-anticoagulant (EPCR) were found in mesothelial cells after treatment with cytokines IL-6 **(B)**, IL-8 **(C)** and growth factors HGF **(D)**, GCP-2 **(E)** by qPCR. The results are expressed as mean ± SEM of six independent experiments. *P<0.05, **P<0.01, compared with the control.

5- Ovarian ascites induce EMT markers in mesothelial cells.

Mesothelial cells in culture medium express basal line amounts of EMT markers such as Snail, Slug, Vimentin, Fibronectin, Twist and N-cadherin (Figure 5A).The basal amount of these markers as well as E-cadherin presented in figure 5B (green colors). When these cells were incubated with 25% ovarian ascites, as presented in figure 5B, all EMT markers, except E-cadherin, were up-regulated. These results indicate the narrow correlation between cell shape change and epithelial mesenchymal transition.

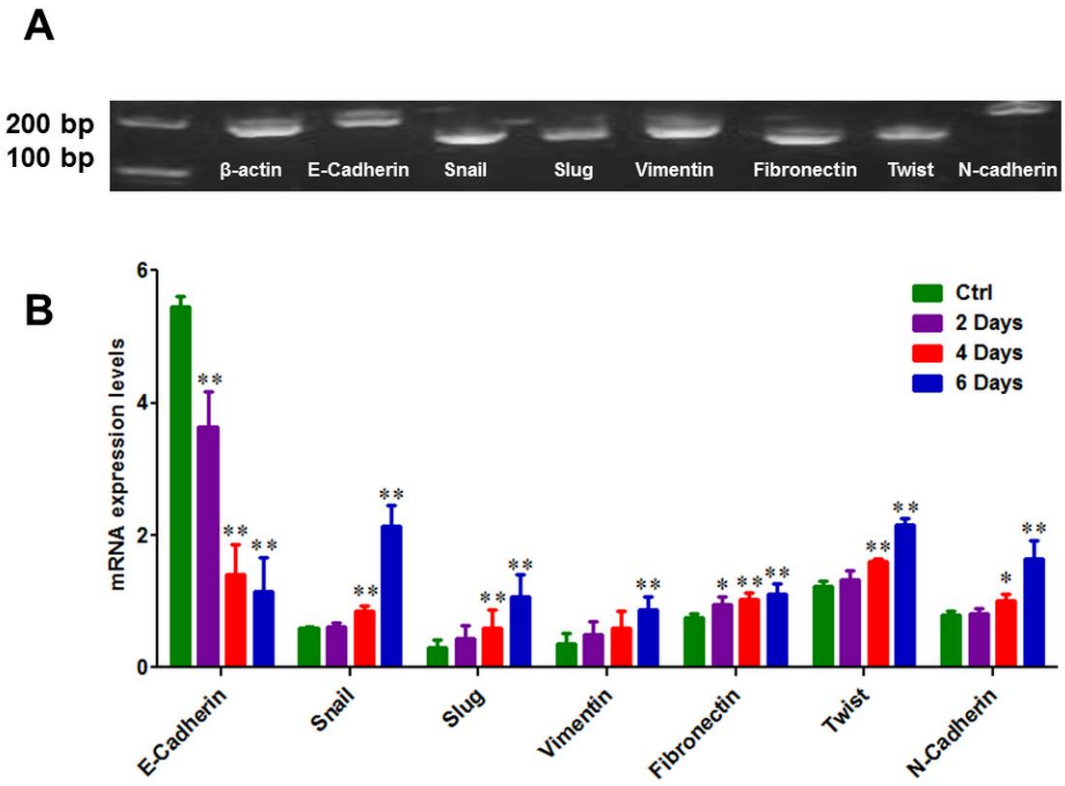


Figure 5. mRNA expression of EMT markers in mesothelial cells:

mRNA of epithelial to mesenchymal (EMT) markers (E-cadherin, Snail, Slug, Vimentin, Fibronectin, Twist and N-cadherin) were detected by RT-PCR **(A),** epithelial marker like E-cadherin was found higher while mesenchymal markers (Snail, Slug, Vimentin, Fibronectin, Twist and N-cadherin) were found lower in

mesothelial cells after treatment with 25% ovarian ascites by qPCR **(B)** The results are expressed as mean ± SEM of six independent experiments. *P<0.05, **P<0.01, compared with the control.

6- Ovarian ascites induces up regulation of proteolytic enzymes and procoagulant factors in mesothelial cells.

The basal mRNA expression of several metalloproteases (neprilysin, MMP2, MMP7 and MMP9), pro-coagulant factors (tissues factor TF) and anti-procoagulant factor (endothelial protein C receptor EPCR) as well as neprilysin activity of the mesothelial cells are presented in figure 6B-D in green colors. When these cells were incubated for 48hrs in culture medium complemented with 25% ovarian ascites, the expression of all markers, except EPCR, were up-regulated. The results presented in figure 6B demonstrate the upregulation of TF and down regulation of EPCR. These results are in support of global pro-coagulant activity of mesothelial cell microenvironments. Among MMPs, the neprilysin, MMP2 and MMP9 were more expressed than MMP7. Neprilysin activity was also found at a higher level. Consequently, ascites–mesothelial cell interaction induces the expression of membrane bound enzyme neprilysin as well as MMP2. When mesothelial microenvironment is modified by carcinomatosis fluids, the high expression of MMPs and neprilysin can be inductor of mesothelial detachment. As presented in figure 6E and 6F, the carcinomatosis cell clusters contain the mesothelial cells stained by D2-40 and WT-1 antibodies respectively.

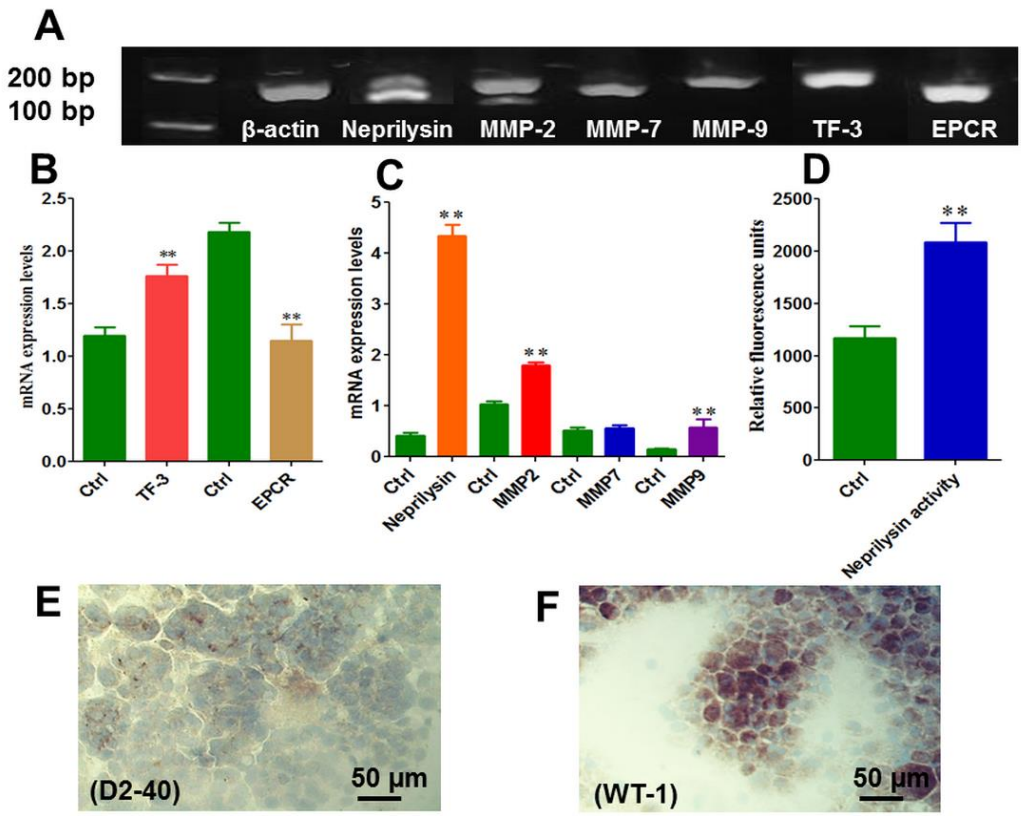


Figure 6. mRNA expression of procoagulant, anti-procoagulant and metalloproteinase markers and neprilysin activity in mesothelial cells as well as immunohistochemistry of ascitic cells from ovarian cancer:

mRNA of procoagulant (TF-3), anti-coagulant (EPCR) and metalloproteinase markers (Neprilysin, MMP-2, MMP-7 and MMP-9) were detected by RT-PCR (A), procoagulation marker (TF-3) was found higher but anti-procoagulant marker (EPCR) lower (B), metalloproteinase and neprilysin were also found higher by qPCR (C) as well as neprilysin activity in mesothelial cells was found higher than control in mesothelial cells after treatment with ovarian ascites (E). Protein expression level of ascitic cells is shown for Wt-1 (F) and D2-40 (G). Pictures were taken at 20× magnification. The results are expressed as mean ± SEM of six independent experiments. *P<0.05, **P<0.01, compared with the control.

7- Cancer cell cluster in carcinomatosis associated with fibrin fiber.

Isolated cancer cell cluster from peritoneal fluid of carcinomatosis are associated with fibrin fiber. As presented in figure 7A and 7B, cell aggregates stained using methylene blue and eosin dyes englobed by fibers. The transmission electron

microscopy of cell cluster presented in (Figure 7C) shows the fibrin network around ~~of~~ the cells (red arrow). High magnification of fibrin reticulation presented in figure 7D indicated the typical fibrin structure. These results suggest that fibrin can be a skeletal structure for the cancer cell cluster integrities.

Our *in vitro* study showed that when the cancer cells were incubated with fibrin clot generated by a pool of several human plasma (Figure 7E), they interacted and adhered to fibrin fiber (Figure 7F). The detail of phyllopod interaction with fibrin is demonstrated in figure 7G (the phyllopod adhered on the fibrin net) and Figure 7H (the phyllopod inserted into the clot).

In parallel studies, the interaction of cancer cells with fibrin clot was analyzed by micro cinematography. Here, when the fibrin clot was incubated with culture medium, the fibrin fibers filaments disintegrated from fibrin clot body in a time dependent manner (Figure 7K).

In this study we distinguished three kinds of interactions; 1) the direct interaction of cells with fibrin clot surface, 2) the interaction of the cells with detached fiber filaments that modified the kinetic of cell interaction with fibrin corpus 3) the inhibition of cells to adhere the fibrin corpus and confining the cancer cells. The data presented in figure 7I shows that after 28hrs of incubation of cancer cells with fibrin clot, they covered the distance 507 µm length within 76 min (zone 1) while the fiber filaments generated from clots have 53 µm lengths (zone 2) (the mean of 3 experiments). In this condition, the detached fiber filaments prevent but cannot inhibit the cancer cell-fibrin clot interaction. 50 minutes are needed for the cancer cells to attach the fibrin clot (speed of cell migration on the clot 0.69 to 1.05 µm/ min (zone 3)). 45hrs later, the detached fiber filaments inhibits the interaction of cancer cells with fibrin clot body and sequestered the cancer cells. Curiously, these fibrin unattached cells generate cells agglomerates and form cancer cell clusters (Figure 7J). The detached fibrin filament along with time presented in figure 2K.

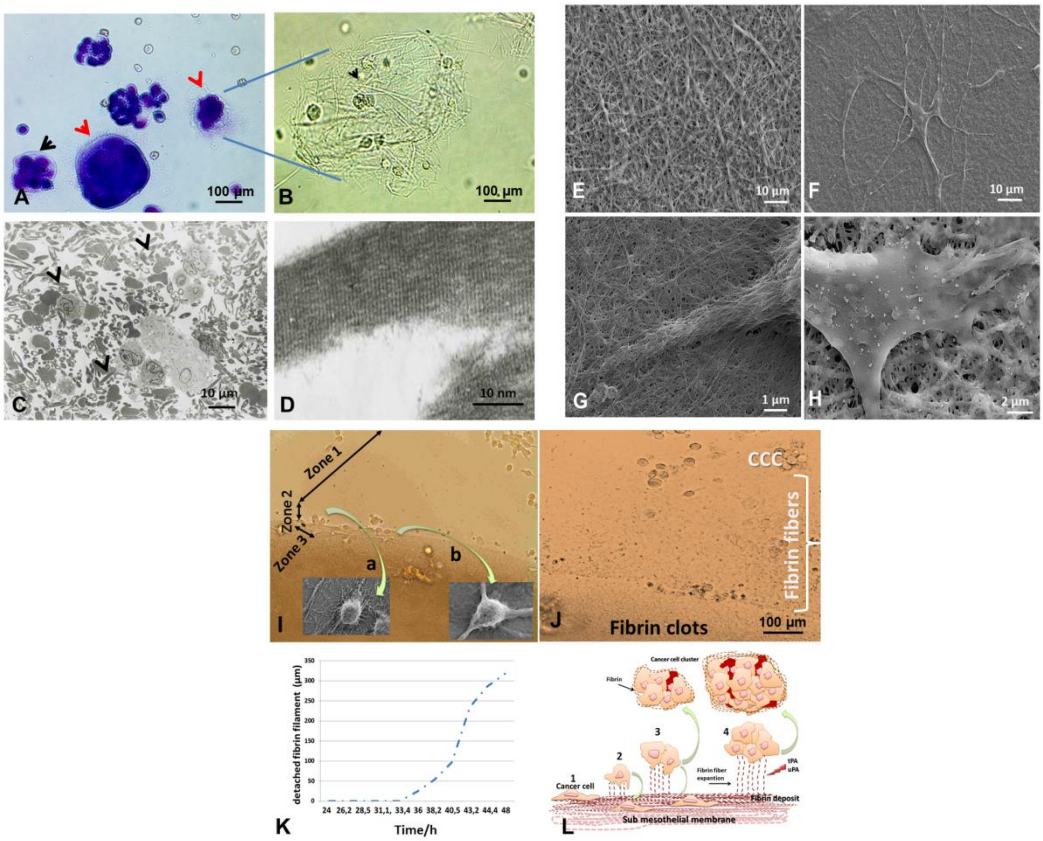


Figure 7. Cancer cell fibrin network interaction:

Shows cell aggregates, isolated from ascites, stained using methylene blue and eosin dyes (A), The fibrin deposit with fiber filaments expansion (red arrow) and without (black arrow) were shown. The cancer cell cluster with fibrin networks (back arrow) of isolated from ascites (initial magnification is x40) (B) and electronic micrographs of ascitic cell clusters (x650) (C), a view of fibrin fibers (x71000) analyzed by transmission electron microscopy (D). Interaction of cancer cells with the fibrin clots, analyzed by scanning electron microscopy (E-H) and by photonic microscopy (I and J picture resulting from micro cinematography analysis, CCC: cancer cell cluster). Graphical representation of detached fibrin filament with time was presented in K. The diagram of different steps for cancer cell cluster formation is shown in (L), urokinase plasminogen activator (uPA) and tissues plasminogen activator (tPA).

Discussion

The natural history of peritoneal carcinomatosis in ovarian cancer is similar to digestive tumor with peritoneum metastasis and dissemination. The late consequences of this phenomenon are cancer nodule formation on the peritoneal surface, ascites

generation and the formation of cancer cell clusters suspended in peritoneal liquid. All these elements contribute to generate the favorable microenvironments for cancer cell growth and dissemination in peritoneal cavity.

The peritoneum, covered by mesothelial cells layer forms a non-adhesive protective surface involving in fluid transport as well as antigen presentation, coagulation and fibrinolysis. In peritoneal cavity, the pathologic conditions lead to peritoneal wound, mesothelial desquamation and cell migration and liberation.

In carcinomatosis, the presence of cancer cells in peritoneal cavity induces the pro-inflammatory state and modifies the mesothelial cell layer microenvironment[578]. The mesothelial layer is disrupted and cells lose their polarity [579].When mesothelial cells are cultured with the ovarian carcinomatosis fluids (1 to 6 days), the cells change their shape and acquire mesenchymal characteristics as reflected by the up regulation of Slug, Snail, Vimentin, N-cadherin, Twist, and Fibronectin (*p=0.001*). E-cadherin, was down-regulated (*p=0.001*). In parallel studies by cytokine array analysis of peritoneal fluid of ovarian, colic and gastric carcinomatosis, we showed high amounts of several family of cytokines such as nerve growth factor (NGF), vascular endothelial growth, (VEGF), macrophage inflammatory proteins (MIF), immune-inflammatory, MMP/TIMP and adiponectin as well as the cytokines and growth factors involved in EMT (results not shown). These observations indicate that mesenchymal transformation of the mesothelial cells is due to favorable microenvironments, generated by carcinomatosis fluids. Upregulation of fibronectin in transformed cells can be a target for cancer cell adhesion.

Carcinomatosis ascites induces cancer cell migration and also considerable changes in mesothelial cytokines secretion. In conditioned supernatants of mesothelial cells, after incubation of these cells in carcinomatosis peritoneal fluid (25%), interleukin 6 (IL6), IL8, MCP-1, GRO, ANG, GCP-2, and hepatocyte growth factor (HGF) were up-regulated. In the same condition, we tested the expression of some metalloproteinases such as MMP-2, MMP-7, MMP-9 and neprilysin known as a membrane metallo-endopeptidase (CD10). Among these enzymes, MMP-2, MMP9 and neprilysin are up-regulated significantly (*p=0.001*) reflecting enhanced extracellular matrix degradation activity. The mRNA expression of neprilysin, MMP 9 and MMP 2 increased10.4 and 1.7 times respectively when mesothelial cells acquired mesenchymal characteristics via EMT phenomena. This result was confirmed by neprilysin activity, examination of biopsies from ovarian carcinomatosis via immuno-histochemical analysis and over

expression of CD10 immunoreactivity in mesothelial cells. Curiously, all peritoneal cells extracted from ascites of ovarian or digestive cancers presented high amount of detached mesothelial cells stained by D2-40 [669] and Wilm's tumor marker (WT-1)[670]. These results are in favor of mesothelial cell detachment in carcinomatous ascites.

When mesothelial cells were incubated with bioactive proteins (IL-6, IL-8, HGF and GCP-2), the upregulation of fibronectin and neprilysin as well as metalloproteinases were observed. HGF and IL-8 modulate adhesion of cancer cells to the peritoneal mesothelium [671, 672]. IL-6 and GCP-2 up-regulate the tissues factor, a pro-coagulant protein [673]. Tissues factors are also up-regulated during mesothelial mesenchymal cell transformation. Interestingly, endothelial protein C receptor (EPCR) is down-regulated under the same condition[674]. The protein C as a natural anticoagulant may participate in non-clotability of carcinomatosis ascites. The increased tissues factor and decreased EPCR expression on transformed mesothelial cells modified the lubrication property of mesothelial cells surface and induced pro-coagulant activity on the peritoneal surface. The immediate consequences of this phenomenon are fibrin formation and deposition. The presence of fibrin on the mesothelial layer of peritoneum in carcinomatosis patients as well as in the carcinomatosis ascites are in agreement with this hypothesis. Our *in vitro* studies show cancer cell adherence and penetration into the fibrin gel when incubated with the plasmatic fibrin clot. These results were confirmed by SEM and microcinematography (MCG) analysis. Findings from SEM pictures related to fibrin and cancer cell interaction indicate the presence of the interfibrillar pores and prolongation of cell philopodes on the gel as well as in the fibrin network. The results were confirmed by MCG in 48 hrs of cancer cell- fibrin gel interaction. The kinetic of cell attachment on the fibrin, in a time dependent manner, showed that attachment occur upto 7hrs and gradually diminishes as fibrin fibers disintegrate and are released. Interestingly, the non-attached cells sequestered by the fibrin fiber in peripheral zone of fibrin clot can agglomerate and divide. In this study we measured the kinetic of fibrin fiber filaments dissociation from fibrin corpus. These results indicate that after fibrinoformation on the internal peritoneum layer, the cancer cells attach to fibrin mass and implant on the peritoneal surface. This fibrin gel in peritoneal fluid can generate the fibrin fiber and inhibit further cancer cell attachment to fibrin corpus. Consequently, the sequestered cells can generate the cancer cells clusters. The

analyses of several MCG supposed that when the cancer cells attached to fibrin corpus, the cells divided and produced a cancer nodule. In contrast the sequestered cells also divided and formed the cell clusters. These clusters may be detached from peritoneal surface by fibrinolytic enzymes such as tissue plasmin activator (tPA) and urokinase (uPA) and liberated in peritoneal fluids [623].

In parallel studies, we observed the shape modification of mesothelial cells, analyzed by SEM on the invasive and non-invasive sample from ovarian carcinomatosis patients. In carcinomatosis zones of peritoneum, compared with non-invasive zone, the mesothelial cells present high deformation and irregularity. The several wounds in mesothelial cell layer suggest the denudation of basal membrane of mesothelium. Morphological aspect also suggests the fibrin fiber deposition in small and vast region on the peritoneal membrane. The fibrin network having the same shape as *in vitro*, covered several zones of peritoneum on the sub mesothelial cells layer in such a way In conclusion the results obtained in this study indicate, for the first time, that cancer nodules develop on the peritoneal membranes due to fibrin formation on the internal peritoneal layer, whereby the cancer cells attach to fibrin mass and implant on the membrane surface. Free fibrin fiber filaments liberated from the fibrin corpus attach to floating cancer cells which agglomerate and form globular cancer cell clusters in the ascitic fluid. These are key events in cancer cell dissemination and carcinomatosis expansion. Our finding is indeed a big stride in understanding the dilemma of peritoneal carcinomatosis.

Disclosures:

The authors have no financial conflicts of interest.

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References

1. Weidle UH, Birzele F, Kollmorgen G, Rueger R. Mechanisms and Targets Involved in Dissemination of Ovarian Cancer. Cancer Genomics-Proteomics **2016**;13(6):407-23.

2. Kenny HA, Chiang C-Y, White EA, Schryver EM, Habis M, Romero IL, *et al.* Mesothelial cells promote early ovarian cancer metastasis through fibronectin secretion. The Journal of clinical investigation **2014**;124(10):4614-28.
3. Cannistra SA. Cancer of the ovary. New England Journal of Medicine **1993**;329(21):1550-9.
4. Davidowitz R, Iwanicki M, Selfors L, Konecny G, Dering J, Slamon D, *et al.* Identification of mechanism involved in mesothelial clearance by ovarian tumor spheroids. AACR; 2011.
5. Slack-Davis J, Atkins K, Harrer C, Hershey D, Conaway M. VCAM-1 regulates ovarian cancer mesothelial invasion and peritoneal metastasis. AACR; 2008.
6. Takatsuki H, Komatsu S, Sano R, Takada Y, Tsuji T. Adhesion of gastric carcinoma cells to peritoneum mediated by α3β1 integrin (VLA-3). Cancer research **2004**;64(17):6065-70.
7. Bourboulia D, Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. 2010. Elsevier. p 161-8.
8. Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. Journal of Biological Chemistry **1989**;264(1):380-8.
9. Honjo K, Munakata S, Tashiro Y, Salama Y, Shimazu H, Eiamboonsert S, *et al.* Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice. The FASEB Journal **2017**;31(6):2625-37.
10. Matte I, Lane D, Laplante C, Garde-Granger P, Rancourt C, Piché A. Ovarian cancer ascites enhance the migration of patient-derived peritoneal mesothelial cells via cMet pathway through HGF-dependent and-independent mechanisms. International journal of cancer **2015**;137(2):289-98.
11. Bekes I, Friedl TW, Köhler T, Möbus V, Janni W, Wöckel A, *et al.* Does VEGF facilitate local tumor growth and spread into the abdominal cavity by suppressing endothelial cell adhesion, thus increasing vascular peritoneal permeability followed by ascites production in ovarian cancer? Molecular cancer **2016**;15(1):13.
12. Mutsaers SE. Mesothelial cells: their structure, function and role in serosal repair. Respirology **2002**;7(3):171-91.
13. Mutsaers SE. The mesothelial cell. The international journal of biochemistry & cell biology **2004**;36(1):9-16.
14. Hassan R, Ho M. Mesothelin targeted cancer immunotherapy. European journal of cancer **2008**;44(1):46-53.
15. Freedman RS, Deavers M, Liu J, Wang E. Peritoneal inflammation–A microenvironment for Epithelial Ovarian Cancer (EOC). Journal of Translational Medicine **2004**;2:23.
16. Sandoval P, Jiménez-Heffernan JA, Rynne-Vidal Á, Pérez-Lozano ML, Gilsanz Á, Ruiz-Carpio V, *et al.* Carcinoma-associated fibroblasts derive from mesothelial cells via mesothelial-to-mesenchymal transition in peritoneal metastasis. The Journal of pathology **2013**;231(4):517-31.
17. Chu AY, Litzky LA, Pasha TL, Acs G, Zhang PJ. Utility of D2-40, a novel mesothelial marker, in the diagnosis of malignant mesothelioma. Modern Pathology **2005**;18(1):105.

18. Amin KM, Litzky LA, Smythe WR, Mooney AM, Morris JM, Mews DJ, *et al.* Wilms' tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma. The American journal of pathology **1995**;146(2):344.
19. Ip CK, Yung S, Chan T-M, Wong AS. Hepatocyte growth factor modulates adhesion of ovarian cancer cells to the peritoneum mesothelium through p70 S6 kinase. AACR; 2011.
20. Kuai W-X, Wang Q, Yang X-Z, Zhao Y, Yu R, Tang X-J. Interleukin-8 associates with adhesion, migration, invasion and chemosensitivity of human gastric cancer cells. World journal of gastroenterology: WJG **2012**;18(9):979.
21. Chin BS, Conway DS, Chung NA, Blann AD, Gibbs CR, Lip GY. Interleukin-6, tissue factor and von Willebrand factor in acute decompensated heart failure: relationship to treatment and prognosis. Blood coagulation & fibrinolysis **2003**;14(6):515-21.
22. Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. British journal of haematology **2001**;115(1):3-12.
23. Buø L, Karlsrud T, Dyrhaug G, Bell H, Engstrom L, Johansen H, *et al.* The fibrinolytic system in human ascites. Scandinavian journal of gastroenterology **1995**;30(11):1101-7.

Complementary document 1.

List of primers sequences and their product size:

Gene	Primers (5`→3`)	Product length (bp)
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	ACCTGAGGCTTTGGATTCCT	
Snail	CCAATCGGAAGCCTAACTACAG	155
	GACAGAGTCCCAGATGAGCATT	
Slug	GCATTTCTTCACTCCGAAGC	151
	TGAATTCCATGCTCTTGCAG	
Vimentin	GAGAACTTTGCCGTTGAAGC	163
	GCTTCCTGTAGGTGGCAATC	
Twist	GTCCGCAGTCTTACGAGGAG	159
	CCAGCTTGAGGGTCTGAATC	
N-Cadherin	CAACTTGCCAGAAAACTCCAGG	205
	ATGAAACCGGGCTATCTGCTC	
Fibronectin	CCGAGGGACCTGGAAGTT	151
	ACTTGCTCCCAGGCACAG	
CD10	ACTGGGAGAAAACATTGCTGAT	183
	TTAACCGCATACTCTGGCCTAT	
MMP-2	ATGACAGCTGCACCACTGAG	174
	ATTTGTTGCCCAGGAAAGTG	
MMP-7	TGCTCACTTCGATGAGGATG	159
	TGGGGATCTCCATTTCCATA	
MMP-9	TTGACAGCGACAAGAAGTGG	174
	TCACGTCGTCCTTATGCAAG	
EPCR	CCTACAACCGCACTCGGTAT	181
	AAGATGCCTACAGCCACACC	
TF-3	GGGCTGACTTCAATCCATGT	195
	GAAGGTGCCCAGAATACCAA	

β-ACTIN	AGAGCTACGAGCTGCCTGAC	184
	AGCACTGTGTTGGCGTACAG	

Complementary document 2.

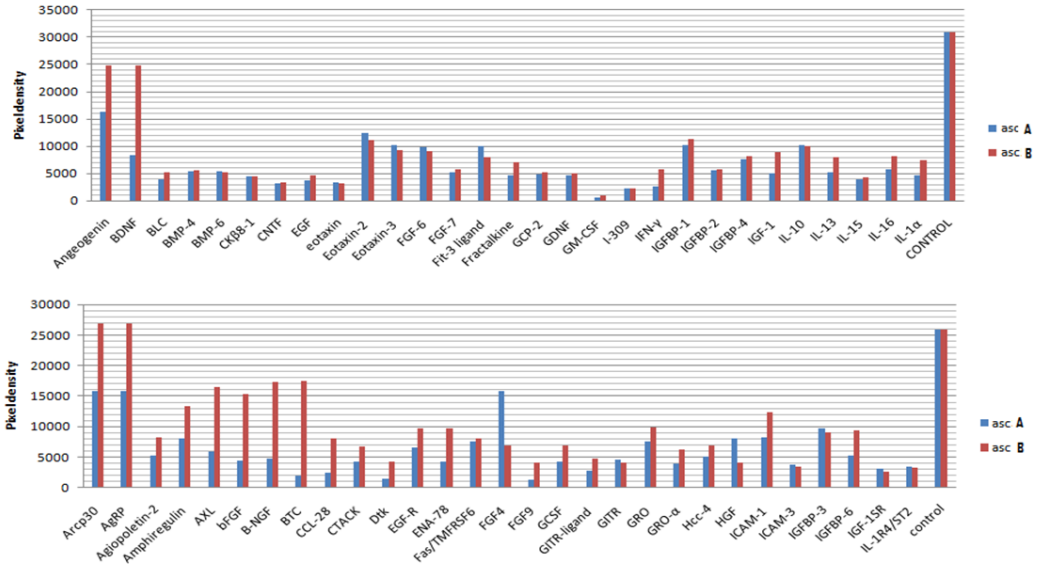
Impact of modified cytokines on mRNA expression level of metalloproteinases, pro-coagulant and pro-anticoagulant:

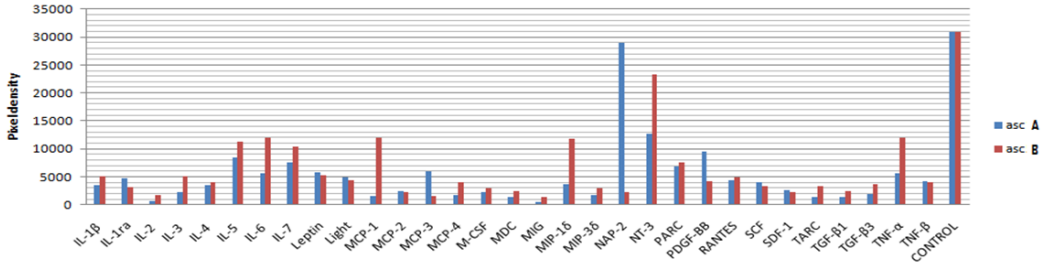
The levels of mRNA folds for metalloproteinases (MMP-2, MMP-7, MMP-9, Neprilysin), procoagulant (TF, FIB) and pro-anticoagulant (EPCR) in mesothelial cells after treatment with IL-6, IL-8, HGF and GCP2.

Mesothelial Treatment	mRNA fold expression						
	MMP-2	MMP-7	MMP-9	Neprilysin	TF	FIB	EPCR
IL-6	1.02	1.97	1.85	1.43	1.21	1.3	0.99
IL-8	1.53	1.04	1.66	1.74	1.02	1.44	1.03
HGF	1.11	1.72	1.04	1.7	0.9	1.38	1.06
GCP-2	1.45	1.9	1.96	3.34	1.18	1.8	1.11

Complementary document 3.

Histograms showing the relative expression of 174 cytokines in two ascites from the patients of ovarian carcinomatosis:

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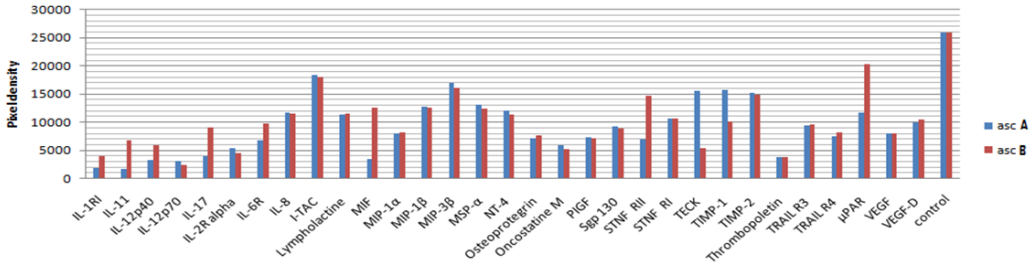
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STNF RII
STNF RI
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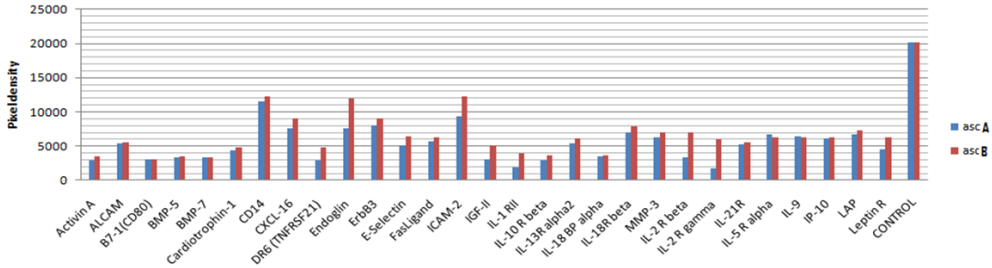
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ErbB3
E-Selectin
FasLigand
ICAM-2
IGF-II
IL-1 RII
IL-10 R beta
IL-13R alpha2
IL-18 BP alpha
IL-18R beta
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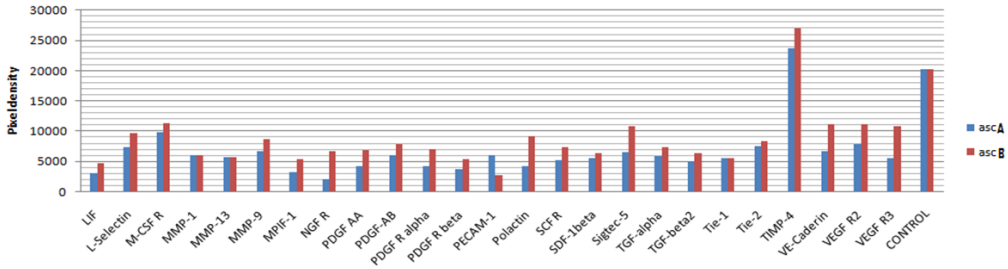
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Article No 4

Thrombopoietin secretion by human ovarian cancer cells

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Research Article

Thrombopoietin Secretion by Human Ovarian Cancer Cells

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The thrombopoietin (TPO) gene expression in human ovary and cancer cells from patients with ovarian carcinomatosis, as well as several cancer cell lines including MDA-MB231 (breast cancer), K562 and HL60 (Leukemic cells), OVCAR-3NIH and SKOV-3 (ovarian cancer), was performed using RT PCR, real-time PCR, and gene sequencing. Human liver tissues are used as controls. The presence of TPO in the cells and its regulation by activated protein C were explored by flow cytometry. TPO content of cell extract as well as plasma of a patient with ovarian cancer was evaluated by ELISA. The functionality of TPO was performed in coculture on the basis of the viability of a TPO-dependent cell line (Ba/F3), MTT assay, and Annexin-V labeling. As in liver, ovarian tissues and all cancer cells lines except the MDA-MB231 express the three TPO-1 (full length TPO), TPO-2 (12 bp deletion), and TPO-3 (116 pb deletion) variants. Primary ovarian cancer cells as well as cancer cell lines produce TPO. The thrombopoietin production by OVCAR-3 increased when cells are stimulated by aPC. OVCAR-3 cell's supernatant can replace exogenous TPO and inhibited TPO-dependent cell line (Ba/F3) apoptosis. The thrombopoietin produced by tumor may have a direct effect on thrombocytosis/thrombosis occurrence in patients with ovarian cancer.

1. Introduction

Thrombosis is a major complication in malignant diseases [1, 2]. More than 50 years ago, Levin and Conley reported that thrombocytosis was associated with breast, lung, digestive, and ovarian cancers [3]. Hemostatic disorders in cancer result from the capacity of tumor cells to secrete procoagulant factors and to interact with blood components such as platelets [4, 5]. While platelets are essential for normal hemostasis, their unbridled activation may result in thrombus formation leading to thrombocytosis complications. Moreover, patients with elevated platelet count have a higher risk to develop venous thromboembolism [6].

Beside their role in coagulation, platelets are also involved in cancer growth and dissemination at different levels [7]. Thus, activated platelets are considered as an important source of lysophosphatidic acid (LPA), which has been shown to be involved in promotion of bone metastasis in a model of mouse bearing breast or ovarian cancer cells [8].

Thrombopoietin (TPO) is a key regulator of megakaryopoiesis and megakaryocyte progenitor proliferation by promoting stem cell differentiation into megakaryocytes and their expansion, hence, boosting platelet production [9, 10].

TPO is mainly produced by the liver and it is also secreted by kidney, bone marrow, and spleen [11]. The human TPO gene is localized on chromosome 3q27 and comprises six exons and five introns [12–14]. To date, 8 different variants of TPO mRNA have been identified, including the full length mRNA (TPO-1) and its 7 alternative splicing variants. Proliferative activity was highlighted only in TPO-1 isoform [15].

Moreover, TPO seems to be more than a megakaryopoiesis regulator. Indeed, TPO has been admitted as a crucial regulator of proliferation and secretory activity in porcine ovarian follicular cells [16]. In pathologic conditions, several cancer cell lines from lung, stomach, liver, and thyroid human carcinomas express the TPO gene [17]. In a case report, Furuhashi et al. reported that TPO could

be produced by ovarian carcinoma [18]. Tsukishiro and his colleagues observed, in a comparative study, that plasma TPO concentration may be a biomarker that distinguishes between benign tumor patients and those with malignant ovarian cancer [19]. It was already described that TPO level increased by an inflammatory process mediated by IL-6, produced by macrophages and monocytes, dose-dependently increases TPO mRNA levels in hepatoma cell lines [20]. However, the presence of thrombopioetin detected by immunohistochemistry is attributed to the capture of thrombopoietin due to thrombopoietin receptor expression in human cancer cells [21].

Previously, we detected TPO release in an adenocarcinoma cell line culture medium (NIH:OVCAR-3 cell line: abbreviated OVCAR-3 in this study). We also observed that activated protein C (aPC), a natural anticoagulant, increased OVCAR-3 TPO secretion [22].

The main goal of this study was to analyze TPO gene expression in ovarian cancer and to assess whether the ovarian TPO produced by cancer cells is functional or not.

2. Materials and Methods

2.1. Cell Culture

2.1.1. Cell Lines. The human cancer cell lines used were ovarian (OVCAR-3 a poorly differentiated serous carcinoma cell line and SKOV-3 an endometrioid cancer cell line), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), leukemic (myeloid leukemia K567 and promyelocytic leukemia HL60), and cervical (HELA). We also used human microvascular endothelial (HMEC-1) and interleukin-3- (IL-3-) dependent murine (Ba/F3) cell lines. Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The Ba/F3 cell line that expresses the human thrombopoietin receptor (MPL) was kindly provided by Caroline Marty and Isabelle Plo [23]. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 50 ug/ml of streptomycin, 50 IU/ml of penicillin, and 2 nM of L-glutamine (Gibco, Saint Aubin, France). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.1.2. Conditioned Media. Cells were seeded in plates or flasks, grown to 80% confluency, and then incubated in serum-free culture medium. Three culture conditions were assayed: (1) in presence of protein C (PC) (Protexel, Courtaboeuf, France) at a concentration of 10 μg/ml, (2) in presence of activated protein C (aPC) (Xigris, Suresnes, France) also at a concentration of 10 μg/ml, and (3) without any addition as control. Cell flasks were incubated 5 hours prior to flow cytometry analysis and cells in plates were incubated 24 hours for coculture experiments. Ba/F3 cell line was cultured in the presence of (1) recombinant IL-3 (5 ng/ml) (Sigma-Aldrich, Saint-Quentin Fallavier, France), (2) human recombinant thrombopoietin (TPO) (50 ng/ml) (LifeSpan Biosciences, USA), (3) PC (10 μg/ml), and (4) aPC (10 μg/ml).

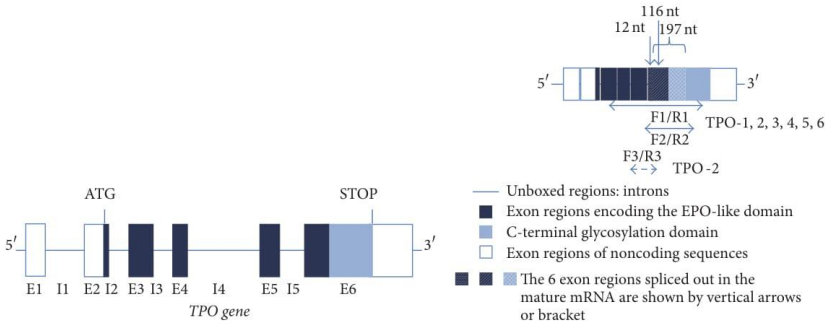
2.1.3. Evaluation of TPO by ELISA. After culture, the cells were carefully washed with phosphate-buffered saline (PBS) and then cells were cultured without fetal calf serum or additional growth factor in culture flask. After 18 hours, the cells were collected and soluble extracts were tested for TPO determination. The TPO was quantified using the commercially available ELISA (R & D Systems Quantikine Human TPO ELISA kit, Abingdon, UK), according to the manufacturer's instructions for cell culture supernatants. The results were expressed in pg/ml/1 × 10^6 cells.

2.1.4. Coculture of Cancer Cells and Ba/F3 Cells. OVCAR-3, MDA-MB23,1 and K562 were cultured in bottom two cell culture compartments separated by a 0.4 μm micropore membrane (Fisher Scientific, Illkirch, France) and Ba/F3 cells were cultured in the upper compartment.

2.1.5. Ascites Cell Culture of Patients. Ascitic fluids from patients were provided by the digestive surgery department of Lariboisiere Hospital (Paris, France). All patients gave their written informed consent. Clinical and biological annotations were recorded in an Access database approved by the "*Commission Nationale de l'informatique et des Libertés,* France." A total of 6 cancer patients were included in the study. Medical records reported that 4 patients had cancer from ovarian origin. Only one patient had a signet ring cell gastric carcinoma. All patients were admitted for surgery during a period from October 2014 to February 2015. Each ascitic sample was centrifuged and the cell pellet obtained was cultured in flasks coated with 0.2% gelatin (Sigma, France) in DMEM medium (GIBCO, Saint Aubin, France), supplemented with 20% of heat-inactivated fetal bovine serum (FBS), 50 ug/ml of streptomycin, 50 IU/ml of penicillin, and 2 nM of L-glutamine.

2.2. Gene Expression

2.2.1. Primers Selection, PCR, and Nested-PCR. TPO primers, for PCR and nested-PCR, were selected from Sasaki et al. study [17]. Our choice was based on TPO gene structure and its possible splice variants as shown in Figures 1(a) and 1(b). Specific primers for TPO were synthetized by Eurofins Genomics (Ebersberg, Germany). Primers used for PCR and nested-PCR are shown in Figure 1(c). The detection of TPO gene expression was investigated by using F1/R1 primers set which amplifies in a common product (all TPO splice variants). We performed also PCR for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure integrity and quantitative comparison of cDNAs. GAPDH cDNA was amplified using specific primers (G1/G2) from Eurofins Genomics (Figure 1(c)). TPO gene expression was investigated by the detection of TPO mRNA in cells from patient's ascites culture or in cell lines. The cell RNA extracts were prepared using the RNeasy kit (Qiagen, Courtabœuf, France). Ovarian total *RNA* and liver *cDNA* from nontumor human adult tissues were provided by BioChain (Newark, USA). Following reverse transcription (Mu-MLV reverse transcriptase and oligo (dT) primers), the polymerase chain reaction (PCR) was performed with MasterMix (5 Prime, Düsseldorf,



Set		Primers	Isoforms: PCR product size (bp)
TPO	F1/R1	F1: 5′-TCCTAACTGCAAGGCTAACG-3′ R1: 5′-AGGGACCTGGAGGTTTGGTT-3′	485–682
	F2/R2	F2: 5′-TGCAGAGCCTCCTTGGAACC-3′ R2: 5′-CTGAATCCCTGCTGCCACTT-3′	TPO-1: 304 TPO-2: 292 TPO-3: 188 TPO-4: 107 TPO-5: 176 TPO-6: 223
	F3/R3	F3: 5′-TTCACCCTTTGCCTACACCT-3′ R3: 5′-GCTGTGGTCCTGCCCTGGGT-3′	TPO-2: 253
GADPH	G1/G2	G1: 5′-ATCATCCCTGCCTCTACTGG-3′ G2: 5′-TGGGTGTCGCTGTTGAAGTC-3′	256

(c)

TaqMan Probes	Reference	Amplicon length (bp)
TPO	Hs01061346_m1	64
GAPDH	Hs02758991_g1	93

(d)

FIGURE 1: TPO primers' selection. (a, b) Schematic illustration of human TPO gene and mRNA isoforms and selected TPO primers. (a) TPO gene contains 6 exons (E 1–6) and 5 introns (I 1–5). (b) Alternative RNA splicing patterns previously identified for TPO. Horizontal arrows represent the amplified regions by RT-PCR. (c) Primers used for PCR and nested-PCR. First PCR products were used as DNA template for the nested-PCR. TPO-amplified isoforms and their sizes are shown. GADPH PCR was used as control. (d) TaqMan Probes for TPO and GADPH.

Germany). The PCR products (using F1/R1 primers, Figure 1), along with a 100 bp DNA ladder, were analyzed by electrophoresis on agarose gels containing GelRed nucleic acid gel stain. After electrophoretic separation of the products, we selectively excised only intense bands for nested-PCR. DNA was eluted and purified using a DNA gel extraction kit (Norgen Biotek Corp, Ontario, Canada). Nested-PCR was performed using F2/R2 or F3/R3 primers and the products were analyzed by agarose gel electrophoresis. The DNA bands of interest were again selected and excised and DNA was purified. Purity and concentration of RNA or DNA samples were determined by optical density measurement and the ratio of 260 : 280 nm using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, France).

2.2.2. Sequencing of Nested-PCR Products. Nested-PCR extracted DNA products were sequenced by Eurofins Genomic (Ebersberg, Germany), using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machine. Sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) in the NCBI database.

2.2.3. Real-Time PCR. TPO gene expression was also analyzed by real-time PCR and TaqMan® primers with FAM probes for TPO or GADPH (Figure 1(d)) were from Applied Biosystems (France). The real-time PCR was performed using TaqMan gene expression assay and LightCycler® 96 real-time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). Analysis of gene expression was performed using the comparative C_T method [24].

2.3. Flow Cytometry

2.3.1. Protein Quantification. OVCAR-3 and MDA-MB231 were cultured in flasks and incubated in a serum-free

medium with or without PC/aPC stimulation as described above. Protein transport inhibitor, containing Brefeldin-A, provided by BD Biosciences (Le Pont de Claix, France), was added (1 μl/ml) or not to the culture medium. Then, cells were detached with accutase, washed with phosphate-buffered saline (PBS), and suspended in 100% heat-inactivated FBS. Cell fixation and permeabilization procedures were performed using the Perfix-nc assay kit as indicated by the manufacturer (Beckman Coulter, France). Cells were mixed at first with an anti-hTPO primary antibody (1 : 200) (Sigma-Aldrich, Saint-Quentin Fallavier, France), then washed with PBS, and incubated with a fluorescein isothiocyanate (FITC) coupled secondary antibody (1 : 100). Cells were then washed twice and the pellet was resuspended in 500 μl of buffer provided in the kit. Flow cytometry was done using a standard Canto II eight-color flow cytometer (Becton Dickinson Biosciences, France) at 530 nm and data analysis was performed using Flowjo software (Flowjo LLC, USA).

2.3.2. Cell Viability. Ba/F3 cells were cocultured separately with MDA-MB231 or OVCAR-3 or K562 cells, in the presence or absence of 10 μg/ml PC or aPC, as indicated above. Ba/F3 cells were cultured with IL-3, TPO, PC, or aPC and served as control. After 72 h, Ba/F3 cells were collected from each well and washed with PBS and then fixed with 4% (final concentration) formaldehyde. Then, Ba/F3 cells were suspended in cold binding buffer (10 mM HEPES pH 7.4, 140 mMNaCl, 2.5 mM CaCl2, 0.1% BSA), incubated for 15 min at 4°C with FITC conjugated Annexin-V (Southern-Biotech, Birmingham, USA), and shielded from light. Data acquisition and analysis were performed by flow cytometry as described above.

2.4. MTT Viability Test. Ba/F3 cells were cocultured with OVCAR-3 or MDA-MB231or K562 cells in the presence of PC or aPC. Ba/F3 cells were cultured with TPO and served as control. After 72 h, Ba/F3 cell viability was investigated using Thiazolyl Blue Tetrazolium Bromide colorimetric assay (MTT) according to Tada et al. protocol [25].

2.5. Statistical Analysis. All values reported are the average ± SEM. Statistical significance was determined using the GraphPad Prism 6.0 software (Kruskal-Wallis test/Student's t-test) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. TPO Expression by Ovarian Carcinomatosis in Primary Culture and Cell Lines. TPO gene expression was analyzed in cultured cells harvested from peritoneal fluids of six patients suffering from carcinomatosis. Clinical characteristics of patients are summarized in Figure 2(a). Photographs taken from each patient cultured cells are presented in Figure 2(b). RT-PCR analysis showed that, unlike the signet ring cells, all the cells from ovarian origin expressed the TPO gene (Figure 2(c)). To extend this analysis, we inspected TPO gene expression in various human cancer cell lines. Results showed different TPO expression with regard to the cell lines considered: ovarian (OVCAR-3 and SKOV-3) and leukemia (K562) cancer cell lines expressed TPO at high level; gastric (AGS, KATO-III), intestinal (LS174T), lung (A549), and cervical (HELA) cancer cell lines expressed TPO at a very low level; TPO gene expression could not be detected in human breast adenocarcinoma (MDA-MB231 and MCF7) and in human microvascular endothelial (HMEC-1) cell lines (Figure 3(a)). TPO gene expression was found to be lower in normal ovary tissue than in the liver.

3.2. Positive TPO Cell Lines Express Different Transcript Patterns and Contained TPO. We next compared the different TPO splice variants in the cancer cell lines that were positive for elevated TPO expression (OVCAR-3, SKOV-3, and K562) and in control ovarian tissues. Results showed that all cancer cells lines express the three TPO-1 (full length TPO), TPO-2 (12 bp deletion), and TPO-3 (116 pb deletion) variants, similarly to the liver and ovary control tissues (Figure 3(b)). Since the splice variants differ by a limited number of nucleotide, the presence of these variants was confirmed by sequencing. Of note, sequencing data showed that the TPO-3 variant harbored the C/T 5183 SNP (Figure 4(a)) which is known to be a common mutation for all clones [14] that does not affect the final sequence of the protein.

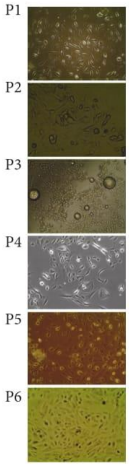
We further quantified the amount of TPO synthetized by ovarian cancer cells OVCAR-3, by the cells from peritoneal carcinomatosis ($n = 22$), as well as leukemic cells K567 and HL60. The MDA cells were used as control. The results presented in Figure 4(b) show that TPO levels in the soluble extract of ovarian cancer are significantly higher than in control.

3.3. Regulation of TPO Production by Activated Protein C. Since we previously showed that aPC-stimulated OVCAR-3 produced TPO by cytokine array [22], we further investigated the regulation of TPO expression by PC and aPC assessed by quantitative PCR in OVCAR-3 before and after stimulation by protein C (PC) or activated protein C (aPC). The results obtained showed that neither PC nor aPC had any effect on TPO mRNA level (Figure 5(a)). To further explore TPO production, we next analyzed TPO protein content in these various condition by flow cytometry. We considered MDA-MB231 cell line as a negative control (Figure 5(b)). We observed that TPO contents of OVCAR-3 cells were similar before and after PC stimulation. However, TPO secretion increased markedly when the cells were incubated with aPC (Figure 5(c)). The results comparing the TPO protein content between OVCAR-3 cells cultured with or without a protein transport inhibitor (Figure 5(d)) allow to conclude that TPO is secreted by the cells.

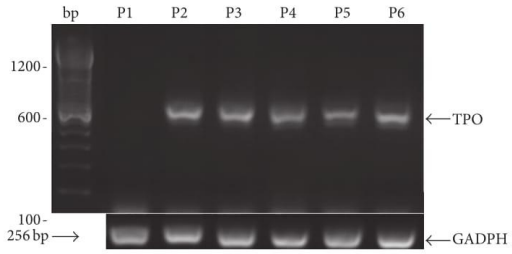
3.4. OVCAR-3 Secretes Functional TPO. To assess whether TPO from OVCAR-3 cells is functional, the TPO-dependent Ba/F3 cell line was cocultured with OVCAR-3, in the presence or absence of PC or aPC. Ba/F3 was cocultured with MDA-MB231 cells that do not produce TPO (as control) or with K562 that produce high level of TPO or cultured alone with PC, aPC, exogenous TPO, or interleukin-3 (IL-3). Ba/F3 cells

Patient	Malignant disease
P1	Signet ring cell carcinoma
P2	Peritoneal carcinomatosis/endometrial cancer/affected ovaries
P3	Peritoneal cancer/ovarian origin
P4	Ovarian cancer
P5	Peritoneal cancer/ovarian origin
P6	Peritoneal carcinomatosis/endometrial cancer/affected ovaries
Mean age	61,2
±SD	7,0

(a)

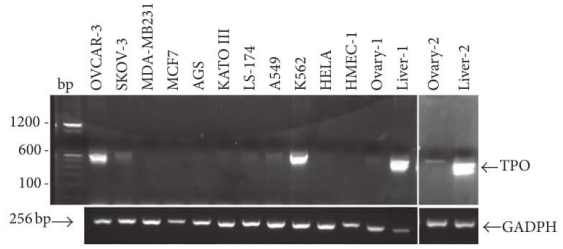


(b)

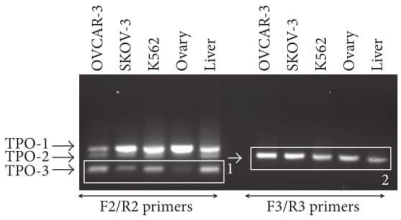


(c)

FIGURE 2: TPO gene expression in cultured cells from ascitic fluids of cancer patients. (a) Subject data. (b) Photographs taken of ascitic fluid cells in culture. (c) Analysis of TPO and GADPH gene expression. 2% agarose gel. PCR using F1/R1 primers for TPO gene amplification.



(a)



(b)

FIGURE 3: TPO gene expression by cell lines. (a) First PCR analysis of TPO and GADPH gene expressed by various cell lines, ovarian (OVCAR-3 and SKOV-3), breast (MDA-MB231 and MCF7), gastric (AGS, KATO-III), intestinal (LSI74T), lung (A549), leukemia (K562), cervical (HELA), and human microvascular endothelial (HMEC-1) cell lines. 2% agarose gel. Normal adult ovary (1 and 2) and liver (1 and 2) tissues served as control. (b) 2% agarose gel pattern of nested-PCR product of TPO: TPO-1 (full length), TPO-2 (12 bp deletion), and TPO-3 (116 bp deletion). Boxes (1, 2) represent bands chosen for sequencing.

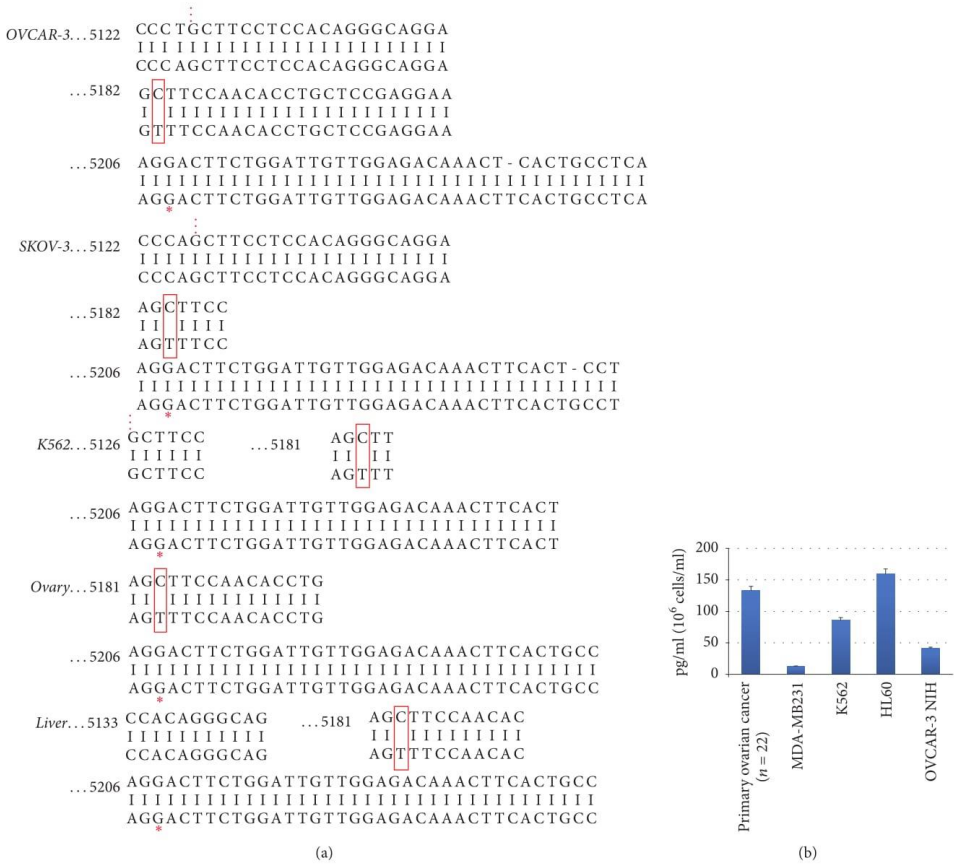


FIGURE 4: Comparison of TPO-3 sequence as well as TPO levels in the soluble cancer cells extracts. (a) Sequences of PCR products extracted from agarose gel bands are shown in the upper line. R2 primer was used for sequencing. Boxes show a C/T 5183 SNP. "..." and "∗" symbols refer to two different alternative splicing sites (12 bp and 116 bp deletions, resp.). A 116 bp deletion characterizing TPO-3 is detected. BLAST reveals no significant mutation. (b) TPO levels in the soluble cancer cells extracts evaluated by enzyme-linked immunosorbent assay (ELISA) for 10^6 cell/ml (the mean of three experiments) from OVCAR-3 NIH cell line, breast cancer MDA-MB231 cell line (as control), myeloid leukemia K562, promyelocytic leukemia HL60 cell lines, and primary ovarian carcinomatosis (n = 22).

viability was assessed in those various conditions by MTT assay and Annexin-V labeling. Viability of Ba/F3 cells in each condition was compared with that of Ba/F3 cells incubated with exogenous TPO. The results showed that the viability of Ba/F3 cells was identical when cocultured with OVCAR-3 cells with or without PC stimulation but increased when cocultured with OVCAR-3 stimulated by aPC.

In addition Ba/F3 also survived when incubated with IL-3 (Figures 6(a) and 6(b)). Using MTT assay, we evaluated the amount of TPO produced by OVCAR-3 stimulated or not by aPC, by comparing the survival of Ba/F3 cells incubated with xenogeneic TPO and cocultured with aPC-stimulated OVCAR-3 (Figure 6(c)). Relative TPO secreted (ng) is indicated in Figure 6(d).

These observations indicated that the TPO secretion via aPC is mediated by guanine nucleotide exchange factor (GBFI).

4. Discussion

The results presented here confirm that normal ovarian tissue as well as ovarian cancer cells expresses TPO and

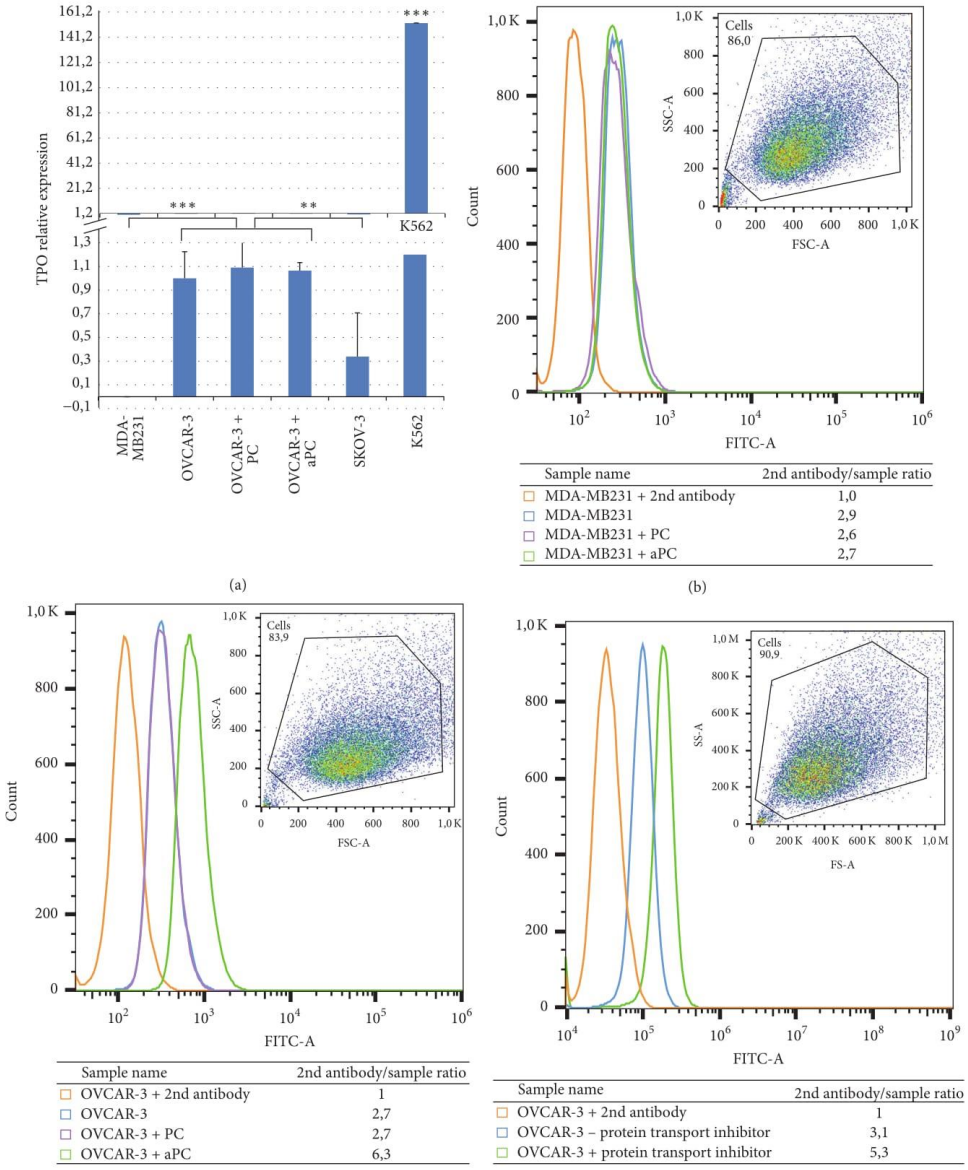
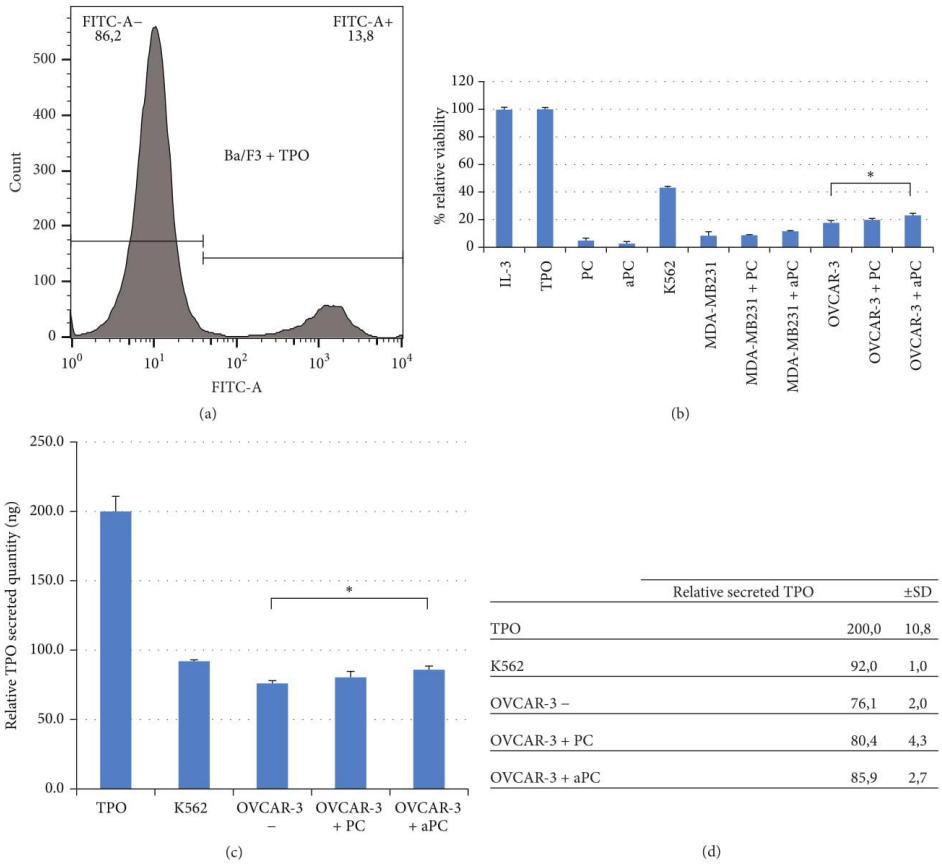


FIGURE 5: TPO gene expression in the presence or absence of protein C. (a) Quantification of TPO gene expression using TaqMan Probes in different cancer cell lines such as MDA-MB231 (as control), OVCAR-3, SKOV-3, and K562. Nonsignificant results were observed in the presence of protein C ($P < 0,05$). High TPO gene expression was observed in leukemic K562 cell. Kruskal-Wallis test ($^{**}P < 0,02$, $^{***}P < 0,01$). Flow cytometry distribution plots are shown. Graphs represent Geometric Fluorescence Mean. (b, c): graphs for MDA-MB231 (b) and OVCAR-3 (c) cells incubated with the secondary antibody alone (orange), labeled with primary and secondary antibodies after incubation without PC or aPC (blue), and with PC (purple) or with aPC (green). (d) TPO-released protein by OVCAR-3. OVCAR-3 were incubated (for 5 hours) without (blue) or with (green) protein transport inhibitor or with only 2nd antibody as control (orange). 2nd antibody GMFI/GMFI sample ratio was calculated for each condition.



	Relative secreted TPO	±SD
TPO	200,0	10,8
K562	92,0	1,0
OVCAR-3 −	76,1	2,0
OVCAR-3 + PC	80,4	4,3
OVCAR-3 + aPC	85,9	2,7

(d)

Figure 6: Functionality of TPO: viability study of TPO-dependent Ba/F3 cells. (a) The panel shows the distribution of two populations of Ba/F3 cells cultured in the presence of TPO: a nonlabeled population FITC-Annexin-V (viability) and a labeled population FITC-Annexin-V (apoptosis). (b) The graph shows Ba/F3 viability (not stained by FITC-Annexin-V). (c, d) Relative TPO secreted quantity (ng). ($P < 0{,}02$). Student's t-test ($^{*}P < 0{,}05$).

show for the first time that TPO produced by cancer cells is functional. These results provide new insight into the relationship between cancer and hemostatic disorders.

Thrombocytosis associated with malignant disease was, traditionally, attributable to interleukin-6 (IL-6) or to granulocyte-macrophage colony-stimulating factor [26–28]. Stone et al. suggested that IL-6 may act alone in a paracrine manner to increase hepatic thrombopoietin production, thereby increasing platelet count [28].

Furthermore, Sakar et al. demonstrated the expression of TPO and its receptor c-MPL in bovine ovarian follicles. They also showed that TPO and c-MPL expression and production in the corpus luteum, during oestrous cycle, vary depending on the luteal stage [29]. Therefore, locally produced TPO and c-MPL may play a crucial role in the regulation of the platelets generation during oestrous cycle.

In addition, TPO serum levels are more elevated in women with ovarian cancer than those with benign ovarian cyst [19, 30]. Here we showed that normal ovarian tissue, ovarian cancer nodule, and the ovarian cancer cell lines, especially OVCAR-3, express TPO. These results are reminiscent from a preliminary observation done in ovarian carcinoma [18].

TPO gene expression in cancer cell lines from different origins also is not identical. Ovarian or leukemic cell line expressed more TPO. Curiously, as observed by RT-PCR and flow cytometry, the amount of the TPO extracted from ovarian cancer nodule is higher that its cell line OVCAR-3

or myeloid leukemia K567 and promyelocytic leukemiaHL60 cell line. In contrast no TPO was extracted from breast cancer cell line (MDA-MB231), compared with ovarian or leukemic cells. Previously we found that when OVCAR-3 cells were incubated with activated protein C, the cancer cell migration was upregulated via MEK-ERK and Rho-GTPase pathway signalization [31] and the amount of secreted TPO in the culture medium detected by cytokine array increased four times [22]. In nonpublished results, we also demonstrated that activated protein C and, to a lesser degree, protein C induced the microparticles release in OVCAR-3 cell line. Here again we observed an upregulation of TPO synthesis in the presence of activated protein C in culture medium. Interestingly, when the cancer cell is preincubated in the presence of aPC with Brefeldin-A, an inhibitor of protein transport from endoplasmic reticulum to golgi apparatus [32, 33], TPO secretion was inhibited. These observations indicated that the TPO secretion via aPC is mediated by guanine nucleotide exchange factor (GBFI).

In parallel studies, the level of the TPO in the plasma of patients with ovarian cancer ($n = 25$) was significantly increased, compared with normal plasma levels of TPO (results not shown).

The sequence analysis of TPO genetic materials in the cell lines confirms that the ovarian cancer cells lines as well as leukemic cells expressed the three TPO-1 (full length TPO), TPO-2 (12 bp deletion), and TPO-3 (116 pb deletion) variants. We do not observe any modification of gene sequences compared with the liver and ovary origin. A TPO-3 variant (C/T 5183 SNP) is known to be a common mutation.

Concerning functional activity analysis of TPO secreted by ovarian cancer cells, we showed that the coculture of ovarian cancer cell line in conditional medium with a TPO-dependent Ba/F3 cell line could decrease the Ba/F3 cell apoptosis due to secretion of TPO from OVCAR-3 cells.

In addition, we report for the first time that the pattern of expression of the TPO gene in ovarian cancer cells is similar to that observed in the liver, and most importantly that the TPO produced is functional.

These results have two major clinical implications.

(i) First, TPO could be used as a biomarker for the detection and progression of ovarian pathology. Indeed, data suggest hypothesis whereby TPO-secreting ovarian cancer cells contribute significantly to the elevation of TPO plasma level in patients with ovarian cancer. Further study should be performed to establish a quantitative relationship between TPO plasma level and cancer progression.

(ii) Second, the production of functional TPO by ovarian cancer cells may be responsible for the risk of thromboembolism or thrombocytosis in patients with ovarian cancer. In such a context, it is most likely that the TPO produced by the cancer cells directly act to promote the expansion of platelets.

Conflicts of Interest

The authors have no financial conflicts of interest.

Acknowledgments

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References

[1] A. Trousseau, *Clinique Médicale de l'Hôtel-Dieu de Paris*, Baillière, 1865.

[2] H. R. Buller, F. F. Van Doormaal, G. L. Van Sluis, and P. W. Kamphuisen, "Cancer and thrombosis: from molecular mechanisms to clinical presentations," *Journal of Thrombosis and Haemostasis*, vol. 5, no. 1, pp. 246–254, 2007.

[3] J. Levin and C. L. Conley, "Thrombocytosis associated with malignant disease," *Archives of Internal Medicine*, vol. 114, no. 4, pp. 497–500, 1964.

[4] M. Z. Wojtukiewicz, E. Sierko, and W. Kisiel, "The role of hemostatic system inhibitors in malignancy," in *Seminars in Thrombosis and Hemostasis*, pp. 621–642, Stratton Intercontinental Medical Book Corporation, New York, NY, USA, 2007.

[5] R. L. Bick, "Coagulation abnormalities in malignancy: a review," *Seminars in Thrombosis and Hemostasis*, vol. 18, no. 4, pp. 353–372, 1992.

[6] A. A. Khorana, C. W. Francis, E. Culakova, and G. H. Lyman, "Risk factors for chemotherapy-associated venous thromboembolism in a prospective observational study," *Cancer*, vol. 104, no. 12, pp. 2822–2829, 2005.

[7] N. M. Bambace and C. E. Holmes, "The platelet contribution to cancer progression," *Journal of Thrombosis and Haemostasis*, vol. 9, no. 2, pp. 237–249, 2011.

[8] A. Boucharaba, C.-M. Serre, S. Grès et al., "Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer," *Journal of Clinical Investigation*, vol. 114, no. 12, pp. 1714–1725, 2004.

[9] F. J. De Sauvage, P. E. Hass, S. D. Spencer et al., "Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand," *Nature*, vol. 369, no. 6481, pp. 533–538, 1994.

[10] D. V. Jones Jr., M. Ashby, S. Vadhan-Raj et al., "Recombinant human thrombopoietin clinical development," *Stem Cells*, vol. 16, pp. 199–206, 1998.

[11] R. Sungaran, B. Markovic, and B. H. Chong, "Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization," *Blood*, vol. 89, no. 1, pp. 101–107, 1997.

[12] Y. Sohma, H. Akahori, N. Seki et al., "Molecular cloning and chromosomal localization of the human thrombopoietin gene," *FEBS Letters*, vol. 353, no. 1, pp. 57–61, 1994.

[13] D. C. Foster, C. A. Sprecher, F. J. Grant et al., "Human thrombopoietin: gene structure, cDNA sequence, expression, and chromosomal localization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 26, pp. 13023–13027, 1994.

[14] A. L. Gurney, W.-J. Kuang, M.-H. Xie, B. E. Malloy, D. L. Eaton, and F. J. De Sauvage, "Genomic structure, chromosomal localization, and conserved alternative splice forms of thrombopoietin," *Blood*, vol. 85, no. 4, pp. 981–988, 1995.

[15] R. Marcucci and M. Romano, "Thrombopoietin and its splicing variants: structure and functions in thrombopoiesis and beyond," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1782, no. 7-8, pp. 427–432, 2008.

[16] A. V. Sirotkin, P. Sanislo, H.-J. Schaeffer et al., "Thrombopoietin regulates proliferation, apoptosis, secretory activity and intracellular messengers in porcine ovarian follicular cells: involvement of protein kinase A," *Journal of Endocrinology*, vol. 183, no. 3, pp. 595–604, 2004.

[17] Y. Sasaki, T. Takahashi, H. Miyazaki et al., "Production of thrombopoietin by human carcinomas and its novel isoforms," *Blood*, vol. 94, no. 6, pp. 1952–1960, 1999.

[18] M. Furuhashi, Y. Miyabe, and H. Oda, "A case of thrombopoietin-producing ovarian carcinoma confirmed by immunohistochemistry," *Gynecologic Oncology*, vol. 74, no. 2, pp. 278–281, 1999.

[19] S. Tsukishiro, N. Suzumori, H. Nishikawa, A. Arakawa, and K. Suzumori, "Preoperative serum thrombopoietin levels are higher in patients with ovarian cancer than with benign cysts," *European Journal of Obstetrics Gynecology and Reproductive Biology*, vol. 140, no. 1, pp. 67–70, 2008.

[20] A. Kaser, G. Brandacher, W. Steurer et al., "Interleukin-6 stimulates thrombopoiesis through thrombopoietin: role in inflammatory thrombocytosis," *Blood*, vol. 98, no. 9, pp. 2720–2725, 2001.

[21] L. Columbyova, M. Loda, and D. T. Scadden, "Thrombopoietin receptor expression in human cancer cell lines and primary tissues," *Cancer Research*, vol. 55, no. 16, pp. 3509–3512, 1995.

[22] D. Azzazene, H. A. Thawadi, H. A. Farsi et al., "Plasma endothelial protein C receptor influences innate immune response in ovarian cancer by decreasing the population of natural killer and TH17 helper cells," *International Journal of Oncology*, vol. 43, no. 4, pp. 1011–1018, 2013.

[23] C. Marty, C. Saint-Martin, C. Pecquet et al., "Germ-line JAK2 mutations in the kinase domain are responsible for hereditary thrombocytosis and are resistant to JAK2 and HSP90 inhibitors," *Blood*, vol. 123, no. 9, pp. 1372–1383, 2014.

[24] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[25] H. Tada, O. Shiho, K.-I. Kuroshima, M. Koyama, and K. Tsukamoto, "An improved colorimetric assay for interleukin 2," *Journal of Immunological Methods*, vol. 93, no. 2, pp. 157–165, 1986.

[26] Z. Estrov, M. Talpaz, G. Mavligit et al., "Elevated plasma thrombopoietic activity in patients with metastatic cancer-related thrombocytosis," *The American Journal of Medicine*, vol. 98, no. 6, pp. 551–558, 1995.

[27] A. Suzuki, T. Takahashi, K. Nakamura et al., "Thrombocytosis in patients with tumors producing colony-stimulating factor," *Blood*, vol. 80, no. 8, pp. 2052–2059, 1992.

[28] R. L. Stone, A. M. Nick, I. A. McNeish et al., "Paraneoplastic thrombocytosis in ovarian cancer," *New England Journal of Medicine*, vol. 366, no. 19, pp. 610–618, 2012.

[29] M. Sarkar, S. Schilffarth, D. Schams, H. H. D. Meyer, and B. Berisha, "The expression of thrombopoietin and its receptor during different physiological stages in the bovine ovary," *Reproduction in Domestic Animals*, vol. 46, no. 5, pp. 757–762, 2011.

[30] P. J. Fielder, A. L. Gurney, E. Stefanich et al., "Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets," *Blood*, vol. 87, no. 6, pp. 2154–2161, 1996.

[31] H. Althawadi, H. Alfarsi, S. Besbes et al., "Activated protein C upregulates ovarian cancer cell migration and promotes unclottability of the cancer cell microenvironment," *Oncology Reports*, vol. 34, no. 2, pp. 603–609, 2015.

[32] C. R. Hutchinson, L. Shu-Wen, A. G. McInnes, and J. A. Walter, "Comparative biochemistry of fatty acid and macrolide antibiotic (brefeldin a). Formation in penicillium brefeldianum," *Tetrahedron*, vol. 39, no. 21, pp. 3507–3513, 1983.

[33] S. Kiontke, L. Langemeyer, A. Kuhlee et al., "Architecture and mechanism of the late endosomal Rab7-like Ypt7 guanine nucleotide exchange factor complex Mon1–Ccz1," *Nature Communications*, vol. 8, Article ID 14034, 2017.

DISCUSSION AND PERSPECTIVES

First of all, we studied a case report in which neuronal signature was demonstrated in the nodule of peritoneal ovarian carcinomatosis. Before treatment, tumor composed of small monomorphic cells with high score of mitosis and hyper vascularization. After treatment, two zones were distinguished in all nodules; a zone with small monomorphic and proliferative cells and mitosis and the other non-proliferative zone without cell mitosis and absence of Ki67 marker. Curiously, all neural markers such as NSA, NCAM (CD56), S100 protein and CHR-A were found in non-proliferated zone. We also observed a dramatically decreased number of immune cells (CD3, CD4, CD8 and CD20) in degenerative, non-proliferative zone. These observations are in favors of lack of immunogenicity of cancer cells when they are differentiated to non-tumor cells. All these results suggest that neuronal markers are associated with non-proliferative characteristic of ovarian cancer cells. Overall, our results indicate that the poorly differentiated cancer cells can be switched in some stress condition such as chemotherapy to well differentiated cells.

After observing the role of chemotherapy in cancer differentiation, it might achieve terminal maturation, leading to reduction of cell proliferation as is seen in the physiologic process of maturation. Use of differentiation agents like all-trans-retinoic acid (ATRA) to reactivate endogenous differentiation programs in cancer cells is a better choice in cancer treatment than conventional chemotherapy with harmful side effects.

Next we focused our study on heparanase expression and its role in EMT, tumor microenvironment and its contribution to tumor evolution toward peritoneal metastasis as well as identification of new therapeutic targets including differentiation therapy. We used gastric signet ring cell adenocarcinoma (SRCA) model for this study.

Gastric signet ring cell adenocarcinoma (SRCA) is unique among gastric carcinoma characterized by isolated or small groups of malignant non-cohesive cells (>50%) containing intracytoplasmic mucin, remarkable fibrosis and rapid invasive progression. SRCA is highly resistant to chemotherapy in comparison to other types

of gastric cancers. Peritoneal metastases are the most frequent type of metastasis in patients with SRCA.

First, we did an attempt to clarify the mechanism of epithelial-mesenchymal transition (EMT) and fibrosis of SRCA of stomach, therapeutic effect of aspirin and suramin in KATO-III cell line and terminal differentiation of cells. Heparanase (HPSE) is a multitasking protein characterized by enzymatic and non-enzymatic activities. Signaling activity of HPSE is achieved by interacting with transmembrane proteins, modulating the activity of factors such as FGF-2 and TGF-β.

We found more HPSE expression in the ascitic samples of the patient with SRCA (n=5) of stomach than that of patient with non-SRCA (n=3) of stomach and colic cancer (n=6). Same results were found when HPSE expression was compared between tumoral sites of SRCA and non-SRCA of stomach (n=11) as well as their peri-tumoral sites too. Furthermore, highly intense signals of HPSE mRNA expression was found in KATO-III (SRCA cell line), OVCAR-3, HMEC-1 and primary cancer cells as compared to SKOV-3, MDA-MB231, MCF7, AGS, LS174T, A549, K562 and HELA. SRCA tissue of stomach showed more mRNA expression of HPSE, pro-fibrotic markers (TGF-β1, FGF-2), VEGF-A, EMT markers (E-cad, Snail, Slug, Vimentin) and fibrotic markers (fibronectin, α-SMA and Collagen-I) than their peri-tumoral sites.

Multi-drug resistance (MDR) remains a great obstacle to effective chemotherapy for gastric cancer. Our present work manifested that LRP and MDR-1 proteins were expressed higher than other resistance proteins (MRP1-5, BCRP) in KATO-III cell line for the first time.

Epidemiological evidence indicates that aspirin and suramin impart greater protection against cancer but the molecular basis for this effect is not fully known. A growing body of evidence has shown that suramin is the inhibitor of an enzyme, heparanase. Here we found that suramin has no antagonist effect in phosphorylation pathway induced by heparanase. Our findings demonstrated that suramin has a considerable degree of inhibition on EMT in KATO-III cell line individually and strong inhibitory effect on proliferation collectively. Generally, differentiation agents tend to have less toxicity than conventional cancer treatments. We found a significant differentiation potential of KATO-III cell line into adipocyte, chondrocyte, osteocyte and neurocytes.

In conclusion, for the first time, we found HPSE in SRCA and its activity might be correlated with EMT, attributed to enhance metastasis and fibrosis in SRCA. Chemoresistance of SRCA may also be one part due to EMT. After finding the involvement of heparanase in cancer progression, various heparanase inhibitors such as aspirin, suramin or suramin analogues can be used to treat cancer.

Then, we concentrated on peritoneal carcinomatosis. The natural history of peritoneal carcinomatosis in ovarian cancer is similar with digestive tumor with peritoneum metastasis and dissemination. The late consequences of this phenomenon are cancer nodule formation on the peritoneal surface, ascites generation and the formation of cancer cell clusters that suspended in peritoneal liquid. All these elements reciprocally generate the favorable microenvironments for cancer cell growth and dissemination in peritoneal cavity.

Several studies are devoted to explain the development of peritoneal carcinomatosis. A study in vitro allowed us to highlight a change in shape and acquired mesenchymal characteristics in mesothelial cells when cultured in ovarian carcinomatosis fluids. Induced cancer cell migration, enormous change in mesothelial cytokines secretions, upregulated metalloprotease including active neprilysin, was also detected. It is also interesting to note that all peritoneal cells extracted from ascites of ovarian or digestive cancer presented high amount of detached mesothelial cell. These results are in favor of mesothelial cell detachment in carcinomatous ascites.
We found that when mesothelial cell were incubated with previously increased cytokines after incubation with peritoneal fluid, the upregulation of fibronectin, neprilysin, metalloproteases and tissues factor, a pro-coagulant protein were observed. Tissues factor also upregulated during mesothelial mesenchymal cell transformation. Interestingly, endothelial protein C receptor (EPCR) down regulated in the same condition. We also found the attachment of cancer cells to fibrin corpus and cell division to form clusters.

Overall results indicated that the tumor microenvironment wobbles between coagulation and anticoagulation. From the beginning of the peritoneal carcinomatosis, a procoagulant microenvironment is established. On one hand, mesothelial cells exfoliate from the peritoneal membrane due to neprilysin activity and acquired mesenchymal characteristics by ascitic fluid. On the other hand, platelets and tissue

factor play a major role in the amplification of hypercoagulability on sub mesothelial surface. Thus tumor cells find fertile land to be adhered to fibrin mass and implant on the peritoneal surface. During implantation, cancer cells also divided and formed the cell clusters. These clusters may be detached from peritoneal surface by fibrinolytic enzyme and liberated in peritoneal fluids.

Identification of all possible cytokines or other specific biological abnormalities (heparanase, IL-6, tissue factor, neprilysin, etc.) involved in peritoneal carcinomatosis opens a new avenue to target multiple factors with a single drug. Although fibrin formation during peritoneal carcinomatosis cannot be inhibited directly but its interaction with cancer cell can be controlled by increasing the non-adhesive characteristics of peritoneal membrane using surfactants such as Icodextrin. Icoddextrin increases peritoneal surface area by providing a barrier to adhesion formation in peritoneal cavity. It will be interesting to see if we plan to target carcinomatosis expansion with differentiation or non-cytotoxic drugs along with Icodextrin through novel drug delivery system like PIPAC (Pressurized intra-peritoneal aerosol chemotherapy). PIPAC is an innovative approach to deliver drugs into the peritoneal cavity as a pressurized normothermic aerosol.

Then we focused our study on the relationship between cancer and hemostatic disorder. Thrombopoietin (TPO) is a key regulator of megakaryopoiesis and megakaryocyte progenitor proliferation by promoting stem cell differentiation into megakaryocytes and their expansion, hence, boosting platelet production. TPO is mainly produced by the liver and it is also secreted by kidney, bone marrow, and spleen.

We found high expression of TPO in ovarian cancer nodule as compared to its cell line OVCAR-3 or K567 (myeloid leukemia) and HL60 cell line (promyelocytic leukemia). In contrast no TPO was extracted from breast cancer cell line (MDA-MB231), compared to ovarian or leukemic cells. Upregulation of TPO synthesis in the presence of activated protein C in culture medium of OVCAR-3 was found. Interestingly, TPO secretion via aPC was found to be mediated by guanine nucleotide exchange factor (GBFI). In parallel studies, the level of the TPO in the plasma of patients with ovarian cancer (n=25) was significantly increased, compared to normal plasma levels. The sequence analysis of TPO genetic materials in the cell lines

confirm that the ovarian cancer cells lines as well as leukemic cells expressed the three TPO-1 (full length TPO), TPO-2 (12bp deletion) and TPO-3 (116pb deletion) variants. A TPO-3 variant (C/T 5183 SNP) is known to be a common mutation.

Next we tried to assess whether the ovarian TPO produced by cancer cells is functional or not. To answer this question, we have targeted TPO-dependent Ba/F3 cells viability. Interestingly we showed that the co-culture of ovarian cancer cell line in conditional medium with Ba/F3 cells could decrease the Ba/F3 cell apoptosis due to secretion of TPO from OVCAR-3 cells.

In addition, we reported for the first time that the pattern of expression of the TPO gene in ovarian cancer cells is similar to that observed in the liver, and most importantly that the TPO produced is functional. The production of functional TPO by ovarian cancer cells may be responsible of the risk of thromboembolism or thrombocytosis in patients with ovarian cancer. A hematologist can help to manage cancer patients with high risk of thrombocytosis and cancer associated thrombosis due to thrombopoietin secretion from ovarian cancer cells

In conclusion, the current findings initiate an understanding

- Differentiation of cancer cells after chemotherapy and different inducer media such as adipocyte, chondrocyte, osteocyte and neurocyte
- Involvement of heparanase in epithelial mesenchymal transition, fibrosis, cancer cell activation, proliferation and cell cycle progression
- Cancer nodules development and cancer cell clusters formation due to fibrin net generation on the peritoneal surfaces and in ascites respectively
- Production of functional TPO by ovarian cancer cells

Output of this work and perspective

Tremendous effort has been made to shed light on peritoneal carcinomatosis. The ideas discussed in our study can be summarized as a set of propositions.

1. After observing the role of chemotherapy as well as inducer media in cancer differentiation, it might achieve terminal maturation, leading to reduction of cell proliferation as is seen in the physiologic process of maturation. Use of differentiation agents like all-trans-retinoic acid (ATRA) to reactivate endogenous differentiation programs in cancer cells is a better choice in cancer treatment than conventional chemotherapy with harmful side effects.
2. After finding the involvement of heparanase in cancer progression, various heparanase inhibitors such as aspirin, suramin or suramin analogues can be used to treat cancer
3. Identification of all possible cytokines or other specific biological abnormalities (heparanase, IL-6, tissue factor, neprilysin, etc.) involved in peritoneal carcinomatosis opens a new avenue to target multiple factors with a single drug
4. Although fibrin formation during peritoneal carcinomatosis cannot be inhibited directly but its interaction with cancer cell can be controlled by increasing the non-adhesive characteristics of peritoneal membrane using surfactants such as Icodextrin.
5. A hematologist can help to manage cancer patients with high risk of thrombocytosis and cancer associated thrombosis due to thrombopoietin secretion from ovarian cancer cells

It will be interesting to see if we plan to target carcinomatosis expansion with differentiation or non-cytotoxic drugs along with Icodextrin through novel drug delivery system like PIPAC (Pressurized intra-peritoneal aerosol chemotherapy).

REFERENCES

1. Sudhakar, A., *History of cancer, ancient and modern treatment methods.* Journal of cancer science & therapy, 2009. **1**(2): p. 1.
2. Torre, L.A., et al., *Global cancer statistics, 2012.* CA: a cancer journal for clinicians, 2015. **65**(2): p. 87-108.
3. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* cell, 2000. **100**(1): p. 57-70.
4. Stewart, B. and C.P. Wild, *World cancer report 2014.* World, 2016.
5. Laur6n, P., *The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma.* Acta Pathol Microbiol Scand, 1965. **64**: p. 31-49.
6. Bosman, F.T., et al., *WHO classification of tumours of the digestive system*. 2010: World Health Organization.
7. Ferley, J., et al., *GLOBOCAN 2012 v1. 0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, Fr Int Agency Res Cancer*. 2013.
8. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012.* European journal of cancer, 2013. **49**(6): p. 1374-1403.
9. Antonioli, D.A. and H. Goldman, *Changes in the location and type of gastric adenocarcinoma.* Cancer, 1982. **50**(4): p. 775-781.
10. Connelly, J.H., et al., *Exophytic signet-ring cell carcinoma of the colorectum.* Archives of pathology & laboratory medicine, 1991. **115**(2): p. 134-136.
11. Frost, A.R., et al., *The significance of signet ring cells in infiltrating lobular carcinoma of the breast.* Archives of pathology & laboratory medicine, 1995. **119**(1): p. 64-68.
12. Jiang, H., et al., *The difference in clinic-pathological features between signet ring cell carcinoma and gastric mucinous adenocarcinoma.* Tumor Biology, 2013. **34**(5): p. 2625-2631.
13. Esaki, Y., R. Hirayama, and K. Hirokawa, *A comparison of patterns of metastasis in gastric cancer by histologic type and age.* Cancer, 1990. **65**(9): p. 2086-2090.
14. Hay, E.D., *Interaction of embryonic cell surface and cytoskeleton with extracellular matrix.* American Journal of Anatomy, 1982. **165**(1): p. 1-12.
15. Uitto, J. and D. Kouba, *Cytokine modulation of extracellular matrix gene expression: relevance to fibrotic skin diseases.* Journal of dermatological science, 2000. **24**: p. S60-S69.
16. Verrecchia, F. and A. Mauviel, *TGF-β and TNF-α: antagonistic cytokines controlling type I collagen gene expression.* Cellular signalling, 2004. **16**(8): p. 873-880.
17. TAKENOSHITA, S., et al., *MUCIN PRODUCTION AND SUBSEQUENT INTERSTITIAL FIBROSIS IN GASTRIC-CANCER WITH SIGNET-RING CELLS.* Oncology reports, 1994. **1**(3): p. 537-541.
18. Mahara, K., et al., *Transforming growth factor beta 1 secreted from scirrhous gastric cancer cells is associated with excess collagen deposition in the tissue.* British journal of cancer, 1994. **69**(4): p. 777.

19. Sarwar, C., et al., *Epithelial ovarian cancer at a cancer hospital in a developing country.* Asian Pac J Cancer Prev, 2006. **7**(4): p. 595-8.
20. Ozols, R.F., et al., *Focus on epithelial ovarian cancer.* Cancer cell, 2004. **5**(1): p. 19-24.
21. Fathalla, M.F., *Incessant ovulation—a factor in ovarian neoplasia?* The Lancet, 1971. **298**(7716): p. 163.
22. Cramer, D.W. and W.R. Welch, *Determinants of Ovarian Cancer Risk. II. Inferences Regarding Pathogenesis 2.* Journal of the National Cancer Institute, 1983. **71**(4): p. 717-721.
23. Riman, T., I. Persson, and S. Nilsson, *Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence.* Clinical endocrinology, 1998. **49**(6): p. 695-707.
24. Testa, J.R., et al., *Spontaneous transformation of rat ovarian surface epithelial cells results in well to poorly differentiated tumors with a parallel range of cytogenetic complexity.* Cancer research, 1994. **54**(10): p. 2778-2784.
25. Feeley, K. and M. Wells, *Precursor lesions of ovarian epithelial malignancy.* Histopathology, 2001. **38**(2): p. 87-95.
26. Medeiros, F., et al., *The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome.* The American journal of surgical pathology, 2006. **30**(2): p. 230-236.
27. Ness, R.B. and C. Cottreau, *Possible role of ovarian epithelial inflammation in ovarian cancer.* Journal of the National Cancer Institute, 1999. **91**(17): p. 1459-1467.
28. Ames, B.N., L.S. Gold, and W.C. Willett, *The causes and prevention of cancer.* Proceedings of the National Academy of Sciences, 1995. **92**(12): p. 5258-5265.
29. Espey, L.L., *Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction.* Biology of reproduction, 1994. **50**(2): p. 233-238.
30. Ness, R.B. and C. Cottreau, *RESPONSE: re: possible role of ovarian epithelial inflammation in ovarian cancer.* Journal of the National Cancer Institute, 2000. **92**(2): p. 163-163.
31. Organization, W.H., *International Agency For Research on Cancer GLOBOCAN 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012*. 2013.
32. Ferlay, J., et al., *Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. GLOBOCAN. 2013; 2012 v1. 0, htt p.* globocan. iarc. fr. Accessed, 2015. **30**.
33. Data, U., *United Nations Population Division-World Population Prospects: The 2006 Revision*. 2008.
34. statistics, C., *Overview of European Union (EU) statistics related to cancer.* http://ec.europa.eu/eurostat/statistics-explained/index.php/Cancer_statistics#Further_Eurostat_information, 2017.
35. Hanahan, D. and L.M. Coussens, *Accessories to the crime: functions of cells recruited to the tumor microenvironment.* Cancer cell, 2012. **21**(3): p. 309-322.

36. Chung, H.W. and J.-B. Lim, *Role of the tumor microenvironment in the pathogenesis of gastric carcinoma.* World journal of gastroenterology: WJG, 2014. **20**(7): p. 1667.
37. Son, B., et al., *The role of tumor microenvironment in therapeutic resistance.* Oncotarget, 2017. **8**(3): p. 3933.
38. Isidoro, C., et al., *The second annual conference of International ovarian cancer consortium and the symposium on tumor microenvironment and therapeutic resistance.* Genes & cancer, 2016. **7**(1-2): p. 7.
39. Zou, W., *Regulatory T cells, tumour immunity and immunotherapy.* Nature Reviews Immunology, 2006. **6**(4): p. 295-307.
40. Restifo, N.P., M.E. Dudley, and S.A. Rosenberg, *Adoptive immunotherapy for cancer: harnessing the T cell response.* Nature Reviews Immunology, 2012. **12**(4): p. 269-281.
41. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* N Engl j Med, 2010. **2010**(363): p. 711-723.
42. Komohara, Y., et al., *Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas.* The Journal of pathology, 2008. **216**(1): p. 15-24.
43. Hasita, H., et al., *Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma.* Cancer science, 2010. **101**(8): p. 1913-1919.
44. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* The Journal of clinical investigation, 2012. **122**(3): p. 787-795.
45. De Palma, M. and C.E. Lewis, *Macrophage regulation of tumor responses to anticancer therapies.* Cancer cell, 2013. **23**(3): p. 277-286.
46. Wu, H., et al., *Tumor-associated macrophages promote angiogenesis and lymphangiogenesis of gastric cancer.* Journal of surgical oncology, 2012. **106**(4): p. 462-468.
47. Mittal, D., et al., *New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape.* Current opinion in immunology, 2014. **27**: p. 16-25.
48. Melero, I., et al., *Evolving synergistic combinations of targeted immunotherapies to combat cancer.* Nature Reviews Cancer, 2015. **15**(8): p. 457-472.
49. Miller, J.F. and M. Sadelain, *The journey from discoveries in fundamental immunology to cancer immunotherapy.* Cancer Cell, 2015. **27**(4): p. 439-449.
50. Quante, M., et al., *Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth.* Cancer cell, 2011. **19**(2): p. 257-272.
51. Sappino, A.P., et al., *Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues.* International journal of cancer, 1988. **41**(5): p. 707-712.
52. Olumi, A.F., et al., *Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium.* Cancer research, 1999. **59**(19): p. 5002-5011.
53. Ishii, G., et al., *Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction.* Biochemical and biophysical research communications, 2003. **309**(1): p. 232-240.

54. Direkze, N.C., et al., *Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts.* Cancer research, 2004. **64**(23): p. 8492-8495.
55. Kojima, Y., et al., *Autocrine TGF-β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts.* Proceedings of the National Academy of Sciences, 2010. **107**(46): p. 20009-20014.
56. Moskovits, N., et al., *p53 Attenuates cancer cell migration and invasion through repression of SDF-1/CXCL12 expression in stromal fibroblasts.* Cancer research, 2006. **66**(22): p. 10671-10676.
57. Aboussekhra, A., *Role of cancer-associated fibroblasts in breast cancer development and prognosis.* Int J Dev Biol, 2011. **55**(7-9): p. 841-849.
58. Kiaris, H., et al., *Evidence for nonautonomous effect of p53 tumor suppressor in carcinogenesis.* Cancer research, 2005. **65**(5): p. 1627-1630.
59. Trimis, G., et al., *Expression of p21waf1/Cip1 in stromal fibroblasts of primary breast tumors.* Human molecular genetics, 2008. **17**(22): p. 3596-3600.
60. Trimboli, A.J., et al., *Pten in stromal fibroblasts suppresses mammary epithelial tumours.* Nature, 2009. **461**(7267): p. 1084-1091.
61. Trimmer, C., et al., *Caveolin-1 and mitochondrial SOD2 (MnSOD) function as tumor suppressors in the stromal microenvironment: a new genetically tractable model for human cancer associated fibroblasts.* Cancer biology & therapy, 2011. **11**(4): p. 383-394.
62. Hill, R., et al., *Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis.* Cell, 2005. **123**(6): p. 1001-1011.
63. Mercier, I., et al., *Human breast cancer-associated fibroblasts (CAFs) show caveolin-1 down-regulation and RB tumor suppressor functional inactivation: implications for the response to hormonal therapy.* Cancer biology & therapy, 2008. **7**(8): p. 1212-1225.
64. Karagiannis, G.S., et al., *Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue.* Molecular Cancer Research, 2012. **10**(11): p. 1403-1418.
65. Mao, Y., et al., *Stromal cells in tumor microenvironment and breast cancer.* Cancer and Metastasis Reviews, 2013. **32**(1-2): p. 303-315.
66. Elenbaas, B. and R.A. Weinberg, *Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation.* Experimental cell research, 2001. **264**(1): p. 169-184.
67. Schulz, W., M. Burchardt, and M. Cronauer, *Molecular biology of prostate cancer.* Molecular human reproduction, 2003. **9**(8): p. 437-448.
68. Untergasser, G., et al., *Profiling molecular targets of TGF-β1 in prostate fibroblast-to-myofibroblast transdifferentiation.* Mechanisms of ageing and development, 2005. **126**(1): p. 59-69.
69. Rajski, M., et al., *IGF-I induced genes in stromal fibroblasts predict the clinical outcome of breast and lung cancer patients.* BMC medicine, 2010. **8**(1): p. 1.
70. Kharaishvili, G., et al., *Collagen triple helix repeat containing 1 protein, periostin and versican in primary and metastatic breast cancer: an immunohistochemical study.* Journal of clinical pathology, 2011: p. jclinpath-2011-200106.

71. Sasaki, H., et al., *Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer.* Breast cancer research and treatment, 2003. **77**(3): p. 245-252.
72. Schmidt-Hansen, B., et al., *Extracellular S100A4 (mts1) stimulates invasive growth of mouse endothelial cells and modulates MMP-13 matrix metalloproteinase activity.* Oncogene, 2004. **23**(32): p. 5487-5495.
73. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* cell, 2011. **144**(5): p. 646-674.
74. Ge, Y.-Z., et al., *Circulating endothelial progenitor cell: a promising biomarker in clinical oncology.* Medical Oncology, 2015. **32**(1): p. 1-10.
75. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-966.
76. Peichev, M., et al., *Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors.* Blood, 2000. **95**(3): p. 952-958.
77. Collino, F., et al., *Epithelial–mesenchymal transition of ovarian tumor cells induces an angiogenic monocyte cell population.* Experimental cell research, 2009. **315**(17): p. 2982-2994.
78. Lyden, D., et al., *Impaired recruitment of bone-marrow–derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth.* Nature medicine, 2001. **7**(11): p. 1194-1201.
79. Folkman, J., *Angiogenesis in cancer, vascular, rheumatoid and other disease.* Nature medicine, 1995. **1**(1): p. 27-30.
80. Folkman, J. *The role of angiogenesis in tumor growth*. in *Seminars in cancer biology*. 1992.
81. Asahara, T. and A. Kawamoto, *Endothelial progenitor cells for postnatal vasculogenesis.* American Journal of Physiology-Cell Physiology, 2004. **287**(3): p. C572-C579.
82. Patenaude, A., J. Parker, and A. Karsan, *Involvement of endothelial progenitor cells in tumor vascularization.* Microvascular research, 2010. **79**(3): p. 217-223.
83. Garcia-Barros, M., et al., *Tumor response to radiotherapy regulated by endothelial cell apoptosis.* Science, 2003. **300**(5622): p. 1155-1159.
84. Goon, P., et al., *Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer.* Neoplasia, 2006. **8**(2): p. 79-88.
85. Asahara, T., A. Kawamoto, and H. Masuda, *Concise review: circulating endothelial progenitor cells for vascular medicine.* Stem cells, 2011. **29**(11): p. 1650-1655.
86. Gao, D., et al., *Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis.* Science, 2008. **319**(5860): p. 195-198.
87. Liby, T.A., et al., *Akt3 controls vascular endothelial growth factor secretion and angiogenesis in ovarian cancer cells.* International Journal of Cancer, 2012. **130**(3): p. 532-543.
88. Su, Y., et al., *The PI3K/Akt pathway upregulates Id1 and integrin α4 to enhance recruitment of human ovarian cancer endothelial progenitor cells.* BMC cancer, 2010. **10**(1): p. 459.
89. Tuttle, T.M., *Technical advances in sentinel lymph node biopsy for breast cancer.* The American surgeon, 2004. **70**(5): p. 407.

90. Leu, A.J., et al., *Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation.* Cancer research, 2000. **60**(16): p. 4324-4327.
91. Neuchrist, C., et al., *Vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 expression in squamous cell carcinomas of the head and neck.* Head & neck, 2003. **25**(6): p. 464-474.
92. Mohammed, R.A.A., et al., *Prognostic significance of vascular endothelial cell growth factors-A,-C and-D in breast cancer and their relationship with angio-and lymphangiogenesis.* British journal of cancer, 2007. **96**(7): p. 1092-1100.
93. Schoppmann, S.F., et al., *VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: impact on lymphangiogenesis and survival.* Surgery, 2006. **139**(6): p. 839-846.
94. Wartiovaara, U., et al., *Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation.* Thrombosis and haemostasis, 1998. **80**(1): p. 171-175.
95. Mäkinen, T., et al., *Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3.* The EMBO journal, 2001. **20**(17): p. 4762-4773.
96. Shibuya, M., *Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases.* Journal of biochemistry, 2013. **153**(1): p. 13-19.
97. Kärpänen, T., et al., *Functional interaction of VEGF-C and VEGF-D with neuropilin receptors.* The FASEB Journal, 2006. **20**(9): p. 1462-1472.
98. Harris, N.C., et al., *Proteolytic processing of vascular endothelial growth factor-D is essential for its capacity to promote the growth and spread of cancer.* The FASEB Journal, 2011. **25**(8): p. 2615-2625.
99. Chung, A.S. and N. Ferrara, *Developmental and pathological angiogenesis.* Annual review of cell and developmental biology, 2011. **27**: p. 563-584.
100. Muller, A., et al., *Involvement of chemokine receptors in breast cancer metastasis.* Nature, 2001. **410**(6824): p. 50-56.
101. Achen, M., G. Mann, and S. Stacker, *Targeting lymphangiogenesis to prevent tumour metastasis.* British journal of cancer, 2006. **94**(10): p. 1355-1360.
102. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-317.
103. Zhu, W., et al., *Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo.* Experimental and molecular pathology, 2006. **80**(3): p. 267-274.
104. Glennie, S., et al., *Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells.* Blood, 2005. **105**(7): p. 2821-2827.
105. Djouad, F., et al., *Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals.* Blood, 2003. **102**(10): p. 3837-3844.
106. Djouad, F., et al., *Earlier onset of syngeneic tumors in the presence of mesenchymal stem cells.* Transplantation, 2006. **82**(8): p. 1060-1066.
107. Fierro, F.A., et al., *Marrow-derived mesenchymal stem cells: role in epithelial tumor cell determination.* Clinical & experimental metastasis, 2004. **21**(4): p. 313-319.

108. Eljaafariab, A., *Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens.* Mechanobiology: Cartilage and Chondrocyte, 2004. **61**: p. 469.
109. Rasmusson, I., et al., *Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells.* Transplantation, 2003. **76**(8): p. 1208-1213.
110. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-1822.
111. Honczarenko, M., et al., *Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors.* Stem cells, 2006. **24**(4): p. 1030-1041.
112. Kinnaird, T., et al., *Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms.* Circulation research, 2004. **94**(5): p. 678-685.
113. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis.* Nature, 2007. **449**(7162): p. 557-563.
114. Rafii, A., et al., *Oncologic trogocytosis of an original stromal cells induces chemoresistance of ovarian tumours.* PLoS One, 2008. **3**(12): p. e3894.
115. Castells, M., et al., *Ovarian ascites-derived Hospicells promote angiogenesis via activation of macrophages.* Cancer letters, 2012. **326**(1): p. 59-68.
116. Benabbou, N., et al., *A subset of bone marrow stromal cells regulate ATP-binding cassette gene expression via insulin-like growth factor-I in a leukemia cell line.* International journal of oncology, 2014. **45**(4): p. 1372-1380.
117. Zhang, X.-Y., et al., *Characteristics and growth patterns of human peritoneal mesothelial cells: comparison between advanced epithelial ovarian cancer and non-ovarian cancer sources.* Journal of the Society for Gynecologic Investigation, 1999. **6**(6): p. 333-340.
118. Kishikawa, T., et al., *Two distinct patterns of peritoneal involvement shown by in vitro and in vivo ovarian cancer dissemination models.* Invasion & metastasis, 1994. **15**(1-2): p. 11-21.
119. Pasquet, M., et al., *Hospicells (ascites-derived stromal cells) promote tumorigenicity and angiogenesis.* International Journal of Cancer, 2010. **126**(9): p. 2090-2101.
120. Hall, B., M. Andreeff, and F. Marini, *The participation of mesenchymal stem cells in tumor stroma formation and their application as targeted-gene delivery vehicles*, in *Bone Marrow-Derived Progenitors*. 2007, Springer. p. 263-283.
121. Cao, H., et al., *Mesenchymal stem cell-like cells derived from human gastric cancer tissues.* Cancer letters, 2009. **274**(1): p. 61-71.
122. Zannettino, A., et al., *Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo.* Journal of cellular physiology, 2008. **214**(2): p. 413-421.
123. Kitadai, Y., *Cancer-stromal cell interaction and tumor angiogenesis in gastric cancer.* Cancer Microenvironment, 2010. **3**(1): p. 109-116.
124. Balamurugan, K., *HIF-1 at the crossroads of hypoxia, inflammation, and cancer.* International journal of cancer, 2015.

125. Semenza, G.L., *Targeting HIF-1 for cancer therapy.* Nature reviews cancer, 2003. **3**(10): p. 721-732.
126. Semenza, G.L., *HIF-1 and tumor progression: pathophysiology and therapeutics.* Trends in molecular medicine, 2002. **8**(4): p. S62-S67.
127. Wong, C., T.L. Wellman, and K.M. Lounsbury, *VEGF and HIF-1α expression are increased in advanced stages of epithelial ovarian cancer.* Gynecologic oncology, 2003. **91**(3): p. 513-517.
128. de Gramont, A., et al. *Targeted agents for adjuvant therapy of colon cancer*. in *Seminars in oncology*. 2006. Elsevier.
129. Darland, D.C. and P.A. D'Amore, *Cell cell interactions in vascular development.* Current topics in developmental biology, 2001. **52**: p. 107-149.
130. Hemler, M.E., *Tetraspanin functions and associated microdomains.* Nature Reviews Molecular Cell Biology, 2005. **6**(10): p. 801-811.
131. Levy, S. and T. Shoham, *The tetraspanin web modulates immune-signalling complexes.* Nature Reviews Immunology, 2005. **5**(2): p. 136-148.
132. Levchenko, A., et al., *Intercellular transfer of P-glycoprotein mediates acquired multidrug resistance in tumor cells.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(6): p. 1933-1938.
133. Yeung, T.-L., et al., *Cellular and molecular processes in ovarian cancer metastasis. A Review in the Theme: Cell and Molecular Processes in Cancer Metastasis.* American Journal of Physiology-Cell Physiology, 2015. **309**(7): p. C444-C456.
134. Lis, R., et al., *Oncologic trogocytosis with Hospicells induces the expression of N-cadherin by breast cancer cells.* International journal of oncology, 2010. **37**(6): p. 1453-1461.
135. Laprise, P., et al., *Phosphatidylinositol 3-kinase controls human intestinal epithelial cell differentiation by promoting adherens junction assembly and p38 MAPK activation.* Journal of Biological Chemistry, 2002. **277**(10): p. 8226-8234.
136. Somasiri, A., et al., *Phosphatidylinositol 3-Kinase is required for adherens junction-dependent mammary epithelial cell spheroid formation.* Differentiation, 2000. **66**(2-3): p. 116-125.
137. Nieman, M.T., et al., *N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression.* The Journal of cell biology, 1999. **147**(3): p. 631-644.
138. Hazan, R.B., et al., *N-cadherin promotes adhesion between invasive breast cancer cells and the stroma.* Cell Communication and Adhesion, 1997. **4**(6): p. 399-411.
139. Shintani, Y., et al., *Collagen I–mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1.* The Journal of cell biology, 2008. **180**(6): p. 1277-1289.
140. Shintani, Y., M.J. Wheelock, and K.R. Johnson, *Phosphoinositide-3 kinase–Rac1–c-Jun NH2-terminal kinase signaling mediates collagen I–induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells.* Molecular Biology of the Cell, 2006. **17**(7): p. 2963-2975.
141. Do, T.-V., et al., *Transforming growth factor-β1, transforming growth factor-β2, and transforming growth factor-β3 enhance ovarian cancer*

metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. Molecular Cancer Research, 2008. **6**(5): p. 695-705.
142. Shintani, Y., et al., *Collagen I promotes epithelial-to-mesenchymal transition in lung cancer cells via transforming growth factor–β signaling.* American journal of respiratory cell and molecular biology, 2008. **38**(1): p. 95-104.
143. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nature immunology, 2002. **3**(11): p. 991-998.
144. Pardoll, D., *Does the immune system see tumors as foreign or self?* Annual review of immunology, 2003. **21**(1): p. 807-839.
145. Martinet, L., et al., *Hospicells derived from ovarian cancer stroma inhibit T-cell immune responses.* International Journal of Cancer, 2010. **126**(9): p. 2143-2152.
146. Mazzoni, A., et al., *Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism.* The Journal of Immunology, 2002. **168**(2): p. 689-695.
147. Bingisser, R.M., et al., *Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway.* The Journal of Immunology, 1998. **160**(12): p. 5729-5734.
148. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nature medicine, 2004. **10**(9): p. 942-949.
149. Sharma, S., et al., *Tumor Cyclooxygenase-2/Prostaglandin E2–dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer.* Cancer research, 2005. **65**(12): p. 5211-5220.
150. Ghiringhelli, F., et al., *CD4+ CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor–β–dependent manner.* The Journal of experimental medicine, 2005. **202**(8): p. 1075-1085.
151. Curti, A., et al., *Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25− into CD25+ T regulatory cells.* Blood, 2007. **109**(7): p. 2871-2877.
152. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: a dynamic niche in cancer progression.* J Cell Biol, 2012. **196**(4): p. 395-406.
153. Frantz, C., K.M. Stewart, and V.M. Weaver, *The extracellular matrix at a glance.* J Cell Sci, 2010. **123**(24): p. 4195-4200.
154. Williams, D.F., *The Williams dictionary of biomaterials*. 1999: Liverpool University Press.
155. Tank, P.W. and J.C.B. Grant, *Grant's dissector*. 2012: Lippincott Williams & Wilkins.
156. Mutsaers, S.E., *The mesothelial cell.* The international journal of biochemistry & cell biology, 2004. **36**(1): p. 9-16.
157. Yáñez-Mó, M., et al., *Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells.* New England Journal of Medicine, 2003. **348**(5): p. 403-413.
158. Cunliffe, W. and P. Sugarbaker, *Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy.* British Journal of Surgery, 1989. **76**(10): p. 1082-1090.

159. Holmdahl, L., *The role of fibrinolysis in adhesion formation.* The European journal of surgery. Supplement.:= Acta chirurgica. Supplement, 1996(577): p. 24-31.
160. KOYAMA, T., et al., *TGF-ss1 secreted by gastric fibroblasts up-regulates CD44H expression and stimulates the peritoneal metastatic ability of scirrhous gastric cancer cells.* International journal of oncology, 2000. **16**(2): p. 355-362.
161. Bamias, A., S. Pignata, and E. Pujade-Lauraine, *Angiogenesis: a promising therapeutic target for ovarian cancer.* Critical reviews in oncology/hematology, 2012. **84**(3): p. 314-326.
162. Tahara, E., *Growth factors and oncogenes in human gastrointestinal carcinomas.* Journal of cancer research and clinical oncology, 1990. **116**(2): p. 121-131.
163. Kiyasu, Y., S. Kaneshima, and S. Koga, *Morphogenesis of peritoneal metastasis in human gastric cancer.* Cancer Research, 1981. **41**(3): p. 1236-1239.
164. Reymond, M.A., *Pleura and Peritoneum: the forgotten organs.* Pleura and Peritoneum, 2016. **1**(1): p. 1-2.
165. *Intraperitoneal Chemotherapy of Peritoneal Carcinomatosis Using Pressurized Aerosol as an Alternative to Liquid Solution: First Evidence for Efficacy.* Ann Surg Oncol, 2014. **21**: p. 553-559.
166. Shen, P., et al., *Cytoreductive surgery and intraperitoneal hyperthermic chemotherapy with mitomycin C for peritoneal carcinomatosis from nonappendiceal colorectal carcinoma.* Annals of Surgical Oncology, 2004. **11**(2): p. 178-186.
167. Jayne, D., *Molecular biology of peritoneal carcinomatosis*, in *Peritoneal Carcinomatosis*. 2007, Springer. p. 21-33.
168. Wisbeck, W.M., E.M. Becher, and A.H. Russell, *Adenocarcinoma of the stomach: autopsy observations with therapeutic implications for the radiation oncologist.* Radiotherapy and Oncology, 1986. **7**(1): p. 13-18.
169. Sperti, C., et al., *Recurrence after resection for ductal adenocarcinoma of the pancreas.* World journal of surgery, 1997. **21**(2): p. 195-200.
170. Ranbarger, K.R., W.D. Johnston, and J.C. Chang, *Prognostic significance of surgical perforation of the rectum during abdominoperineal resection for rectal carcinoma.* The American Journal of Surgery, 1982. **143**(2): p. 186-188.
171. Slanetz, C.A., *The effect of inadvertent intraoperative perforation on survival and recurrence in colorectal cancer.* Diseases of the colon & rectum, 1984. **27**(12): p. 792-797.
172. Günther, K., et al., *Prediction of Distant Metastases after Curative Surgery for Rectal Cancer.* Journal of Surgical Research, 2002. **1**(103): p. 68-78.
173. Vegas, H., et al., *Disseminated and circulating tumor cells in gastrointestinal oncology.* Bulletin du cancer, 2012. **99**(5): p. 535-544.
174. Kwakman, R., et al., *Molecular biology of peritoneal carcinomatosis*, in *Intraperitoneal Cancer Therapy: Principles and Practice*. 2015, CRC Press. p. 61-69.
175. Kimura, A., et al., *Peritoneal mesothelial cell injury factors in rat cancerous ascites.* Cancer research, 1985. **45**(9): p. 4330-4333.

176. Iwanicki, M.P., et al., *Ovarian cancer spheroids use myosin-generated force to clear the mesothelium.* Cancer discovery, 2011. **1**(2): p. 144-157.
177. YUTAKAYONEMURA, Y.E., et al., *A possible role of cytokines in the formation of peritoneal dissemination.* International journal of oncology, 1997. **11**: p. 349-358.
178. Witz, C.A., I.A. Monotoya-Rodriguez, and R.S. Schenken, *Whole explants of peritoneum and endometrium: a novel model of the early endometriosis lesion.* Fertility and sterility, 1999. **71**(1): p. 56-60.
179. Niedbala, M.J., K. Crickard, and R.J. Bernacki, *Interactions of human ovarian tumor cells with human mesothelial cells grown on extracellular matrix: an in vitro model system for studying tumor cell adhesion and invasion.* Experimental cell research, 1985. **160**(2): p. 499-513.
180. Hirofumi, K., et al., *A scanning electron microscopic study on the peritoneal implantation of ascites hepatoma AH100B cells in rats.* GANN Japanese Journal of Cancer Research, 1980. **71**(1): p. 8-13.
181. Jiang, C.-G., et al., *Connective tissue growth factor is a positive regulator of epithelial–mesenchymal transition and promotes the adhesion with gastric cancer cells in human peritoneal mesothelial cells.* Cytokine, 2013. **61**(1): p. 173-180.
182. Schlaeppi, M., et al., *Role of integrins and evidence for two distinct mechanisms mediating human colorectal carcinoma cell interaction with peritoneal mesothelial cells and extracellular matrix.* Cell Communication & Adhesion, 1997. **4**(6): p. 439-455.
183. Yonemura, Y., et al., *Inhibition of peritoneal dissemination in human gastric cancer by MMP-7-specific antisense oligonucleotide.* Journal of experimental & clinical cancer research: CR, 2001. **20**(2): p. 205-212.
184. Hay, E., *An overview of epithelio-mesenchymal transformation.* Cells Tissues Organs, 1995. **154**(1): p. 8-20.
185. Micalizzi, D.S., S.M. Farabaugh, and H.L. Ford, *Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression.* Journal of mammary gland biology and neoplasia, 2010. **15**(2): p. 117-134.
186. Thiery, J.P., et al., *Epithelial-mesenchymal transitions in development and disease.* cell, 2009. **139**(5): p. 871-890.
187. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* The Journal of clinical investigation, 2009. **119**(6): p. 1420-1428.
188. De Craene, B. and G. Berx, *Regulatory networks defining EMT during cancer initiation and progression.* Nature Reviews Cancer, 2013. **13**(2): p. 97-110.
189. Lamouille, S., J. Xu, and R. Derynck, *Molecular mechanisms of epithelial–mesenchymal transition.* Nature reviews Molecular cell biology, 2014. **15**(3): p. 178-196.
190. Huang, L., R.-L. Wu, and A.-M. Xu, *Epithelial-mesenchymal transition in gastric cancer.* American journal of translational research, 2015. **7**(11): p. 2141.
191. Yu, M., et al., *Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition.* science, 2013. **339**(6119): p. 580-584.

192. Elloul, S., et al., *Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma.* Cancer, 2005. **103**(8): p. 1631-1643.
193. Takeichi, M., *Cadherins: a molecular family important in selective cell-cell adhesion.* Annual review of biochemistry, 1990. **59**(1): p. 237-252.
194. Takeichi, M., *Cadherins in cancer: implications for invasion and metastasis.* Current opinion in cell biology, 1993. **5**(5): p. 806-811.
195. Tang, A., et al., *Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin.* Nature, 1993. **361**(6407): p. 82.
196. Handschuh, G., et al., *Tumour-associated E-cadherin mutations alter cellular morphology, decrease cellular adhesion and increase cellular motility.* Oncogene, 1999. **18**(30).
197. Singhai, R., et al., *E-Cadherin as a diagnostic biomarker in breast cancer.* N Am J Med Sci, 2011. **3**(5): p. 227-233.
198. Elloul, S., et al., *Expression of E-cadherin transcriptional regulators in ovarian carcinoma.* Virchows Archiv, 2006. **449**(5): p. 520-528.
199. Berx, G., et al., *E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers.* The EMBO journal, 1995. **14**(24): p. 6107.
200. Vos, C., et al., *E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis.* British journal of cancer, 1997. **76**(9): p. 1131.
201. Kanai, Y., et al., *Point Mutation of the E–Cadherin Gene in Invasive Lobular Carcinoma of the Breast.* Cancer Science, 1994. **85**(10): p. 1035-1039.
202. Vleminckx, K., et al., *Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell, 1991. **66**(1): p. 107-119.
203. Thiery, J.P., *Epithelial–mesenchymal transitions in tumour progression.* Nature Reviews Cancer, 2002. **2**(6): p. 442-454.
204. Satelli, A. and S. Li, *Vimentin in cancer and its potential as a molecular target for cancer therapy.* Cellular and molecular life sciences, 2011. **68**(18): p. 3033-3046.
205. Fuyuhiro, Y., et al., *Clinical significance of vimentin-positive gastric cancer cells.* Anticancer research, 2010. **30**(12): p. 5239-5243.
206. Takemura, K., et al., *Expression of vimentin in gastric cancer: a possible indicator for prognosis.* Pathobiology, 1994. **62**(3): p. 149-154.
207. Fritz, G. and B. Kaina, *Rho GTPases: promising cellular targets for novel anticancer drugs.* Current cancer drug targets, 2006. **6**(1): p. 1-14.
208. Lambrechts, A., M. Van Troys, and C. Ampe, *The actin cytoskeleton in normal and pathological cell motility.* The international journal of biochemistry & cell biology, 2004. **36**(10): p. 1890-1909.
209. Tonino, P. and C. Abreu, *Microvessel density is associated with VEGF and α-SMA expression in different regions of human gastrointestinal carcinomas.* Cancers, 2011. **3**(3): p. 3405-3418.
210. Pankov, R. and K.M. Yamada, *Fibronectin at a glance.* Journal of cell science, 2002. **115**(20): p. 3861-3863.
211. Han, S., F.R. Khuri, and J. Roman, *Fibronectin stimulates non–small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signal pathways.* Cancer research, 2006. **66**(1): p. 315-323.

212. David, L., et al., *Expression of laminin, collagen IV, fibronectin, and type IV collagenase in gastric carcinoma.* Cancer, 1994. **73**(3): p. 518-527.
213. Grigioni, W., et al., *Behaviour of basement membrane antigens in gastric and colorectal cancer: immunohistochemical study.* Pathology International, 1986. **36**(2): p. 173-184.
214. Li, Y., et al., *Fibronectin increases RhoA activity through inhibition of PKA in the human gastric cancer cell line SGC-7901.* Mol Med Report, 2011. **4**(1): p. 65-69.
215. Hynes, R.O., *Fibronectins*. 2012: Springer Science & Business Media.
216. Tas, F., et al., *Levels of serum fibronectin as a biomarker in gastric cancer patients: Correlation with clinical diagnosis and outcome.* Molecular and clinical oncology, 2016. **4**(4): p. 655-659.
217. Jerhammar, F., et al., *Fibronectin 1 is a potential biomarker for radioresistance in head and neck squamous cell carcinoma.* Cancer biology & therapy, 2010. **10**(12): p. 1244-1251.
218. Iwatsuki, M., et al., *Epithelial–mesenchymal transition in cancer development and its clinical significance.* Cancer science, 2010. **101**(2): p. 293-299.
219. Jin, H., et al., *Snail is critical for tumor growth and metastasis of ovarian carcinoma.* International journal of cancer, 2010. **126**(9): p. 2102-2111.
220. Yanagawa, J., et al., *Snail promotes CXCR2 ligand–dependent tumor progression in non–small cell lung carcinoma.* Clinical Cancer Research, 2009. **15**(22): p. 6820-6829.
221. Kosaka, T., et al., *Expression of snail in upper urinary tract urothelial carcinoma: prognostic significance and implications for tumor invasion.* Clinical Cancer Research, 2010. **16**(23): p. 5814-5823.
222. Muenst, S., et al., *Nuclear expression of snail is an independent negative prognostic factor in human breast cancer.* Disease markers, 2013. **35**(5): p. 337-344.
223. Yang, M.H., et al., *Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma.* Hepatology, 2009. **50**(5): p. 1464-1474.
224. Kim, M.A., et al., *Prognostic importance of epithelial–mesenchymal transition-related protein expression in gastric carcinoma.* Histopathology, 2009. **54**(4): p. 442-451.
225. Ryu, H.S., et al., *Combination of epithelial-mesenchymal transition and cancer stem cell–like phenotypes has independent prognostic value in gastric cancer.* Human pathology, 2012. **43**(4): p. 520-528.
226. Shin, N.R., et al., *Overexpression of Snail is associated with lymph node metastasis and poor prognosis in patients with gastric cancer.* BMC cancer, 2012. **12**(1): p. 521.
227. Nieto, M.A., et al., *Control of cell behavior during vertebrate development by Slug, a zinc finger gene.* Science, 1994. **264**(5160): p. 835-840.
228. Hajra, K.M., D.Y. Chen, and E.R. Fearon, *The SLUG zinc-finger protein represses E-cadherin in breast cancer.* Cancer research, 2002. **62**(6): p. 1613-1618.
229. Bolós, V., et al., *The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison*

with Snail and E47 repressors. Journal of cell science, 2003. **116**(3): p. 499-511.
230. Alves, C.C., et al., *Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers.* Front Biosci, 2009. **14**(1): p. 3035-3050.
231. Alves, C.C., et al., *Slug is overexpressed in gastric carcinomas and may act synergistically with SIP1 and Snail in the down-regulation of E-cadherin.* The Journal of pathology, 2007. **211**(5): p. 507-515.
232. Cole, W.G., *Collagen genes: mutations affecting collagen structure and expression.* Progress in nucleic acid research and molecular biology, 1994. **47**: p. 29-80.
233. Kita, Y., et al., *Clinical significance of LAMB3 and COL7A1 mRNA in esophageal squamous cell carcinoma.* European Journal of Surgical Oncology (EJSO), 2009. **35**(1): p. 52-58.
234. Wu, Y., et al., *COL11A1 promotes tumor progression and predicts poor clinical outcome in ovarian cancer.* Oncogene, 2014. **33**(26): p. 3432-3440.
235. Ramaswamy, S., et al., *A molecular signature of metastasis in primary solid tumors.* Nature genetics, 2003. **33**(1): p. 49-54.
236. De Caceres, I.I., et al., *Identification of novel target genes by an epigenetic reactivation screen of renal cancer.* Cancer research, 2006. **66**(10): p. 5021-5028.
237. Hayashi, M., et al., *Identification of the collagen type 1 alpha 1 gene (COL1A1) as a candidate survival-related factor associated with hepatocellular carcinoma.* BMC cancer, 2014. **14**(1): p. 108.
238. Bonazzi, V.F., et al., *Cross-platform array screening identifies COL1A2, THBS1, TNFRSF10D and UCHL1 as genes frequently silenced by methylation in melanoma.* PloS one, 2011. **6**(10): p. e26121.
239. Nagathihalli, N.S. and N.B. Merchant, *Src-mediated regulation of E-cadherin and EMT in pancreatic cancer.* Frontiers in bioscience (Landmark edition), 2011. **17**: p. 2059-2069.
240. Derksen, P.W., et al., *Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis.* Cancer cell, 2006. **10**(5): p. 437-449.
241. Peinado, H., D. Olmeda, and A. Cano, *Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?* Nature Reviews Cancer, 2007. **7**(6): p. 415-428.
242. MacDonald, B.T., K. Tamai, and X. He, *Wnt/β-catenin signaling: components, mechanisms, and diseases.* Developmental cell, 2009. **17**(1): p. 9-26.
243. Batlle, E., et al., *The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.* Nature cell biology, 2000. **2**(2): p. 84-89.
244. Adam, L., et al., *miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy.* Clinical Cancer Research, 2009. **15**(16): p. 5060-5072.
245. Morin, P.J., *Drug resistance and the microenvironment: nature and nurture.* Drug Resistance Updates, 2003. **6**(4): p. 169-172.
246. Housman, G., et al., *Drug resistance in cancer: an overview.* Cancers, 2014. **6**(3): p. 1769-1792.

247. Ullah, M.F., *Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy.* Asian Pac J Cancer Prev, 2008. **9**(1): p. 1-6.
248. Leonard, G.D., T. Fojo, and S.E. Bates, *The role of ABC transporters in clinical practice.* The oncologist, 2003. **8**(5): p. 411-424.
249. Choi, C.-H., *ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal.* Cancer cell international, 2005. **5**(1): p. 30.
250. Moitra, K., H. Lou, and M. Dean, *Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development.* Clinical pharmacology and therapeutics, 2011. **89**(4): p. 491.
251. Ozben, T., *Mechanisms and strategies to overcome multiple drug resistance in cancer.* FEBS letters, 2006. **580**(12): p. 2903-2909.
252. Podolski-Renić, A., et al., *The role of paclitaxel in the development and treatment of multidrug resistant cancer cell lines.* Biomedicine & Pharmacotherapy, 2011. **65**(5): p. 345-353.
253. Abolhoda, A., et al., *Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin.* Clinical Cancer Research, 1999. **5**(11): p. 3352-3356.
254. Doyle, L.A., et al., *A multidrug resistance transporter from human MCF-7 breast cancer cells.* Proceedings of the National Academy of Sciences, 1998. **95**(26): p. 15665-15670.
255. Zhang, F., et al., *MDM2 antagonist nutlin-3a reverses mitoxantrone resistance by inhibiting breast cancer resistance protein mediated drug transport.* Biochemical pharmacology, 2011. **82**(1): p. 24-34.
256. Maliepaard, M., et al., *Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line.* Cancer research, 1999. **59**(18): p. 4559-4563.
257. Robey, R.W., et al., *ABCG2: determining its relevance in clinical drug resistance.* Cancer and Metastasis Reviews, 2007. **26**(1): p. 39-57.
258. Cole, S., et al., *Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.* SCIENCE-NEW YORK THEN WASHINGTON-, 1992. **258**: p. 1650-1650.
259. Fardel, O., et al., *Physiological, pharmacological and clinical features of the multidrug resistance protein 2.* Biomedicine & Pharmacotherapy, 2005. **59**(3): p. 104-114.
260. Scheffer, G.L., et al., *Lung resistance-related protein/major vault protein and vaults in multidrug-resistant cancer.* Current opinion in oncology, 2000. **12**(6): p. 550-556.
261. Li, J., et al., *Expression of MRP1, BCRP, LRP, and ERCC1 in advanced non–small-cell lung cancer: correlation with response to chemotherapy and survival.* Clinical lung cancer, 2009. **10**(6): p. 414-421.
262. Berger, W., L. Elbling, and M. Micksche, *Expression of the major vault protein LRP in human non-small-cell lung cancer cells: Activation by short-term exposure to antineoplastic drugs.* International journal of cancer, 2000. **88**(2): p. 293-300.
263. Gupta, S. and S. Gollapudi, *P-glycoprotein (MDR 1 gene product) in cells of the immune system: its possible physiologic role and alteration in aging and human immunodeficiency virus-1 (HIV-1) infection.* Journal of clinical immunology, 1993. **13**(5): p. 289-301.

264. Ludescher, C., et al., *Differential activity of P-glycoprotein in normal blood lymphocyte subsets.* British journal of haematology, 1998. **101**(4): p. 722-727.
265. Puddu, P., et al., *Interferon-gamma up-regulates expression and activity of P-glycoprotein in human peripheral blood monocyte-derived macrophages.* Laboratory investigation; a journal of technical methods and pathology, 1999. **79**(10): p. 1299-1309.
266. Kartner, N., et al., *Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein.* Cancer research, 1983. **43**(9): p. 4413-4419.
267. Mickisch, G.H., et al., *Monoclonal antibody MRK16 reverses the multidrug resistance of multidrug-resistant transgenic mice.* Cancer research, 1992. **52**(16): p. 4427-4432.
268. Hamada, H. and T. Tsuruo, *Functional role for the 170-to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies.* Proceedings of the National Academy of Sciences, 1986. **83**(20): p. 7785-7789.
269. Scheper, R.J., et al., *Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance.* Cancer Research, 1993. **53**(7): p. 1475-1479.
270. IZQUIERDO, M.A., M.L. SLOVAK, and H.M. PINEDOZ, *The drug resistance-related protein LRP is the human major vault protein.* Nature medicine, 1995. **1**(6).
271. Filipits, M., et al., *Clinical relevance of the lung resistance protein in diffuse large B-cell lymphomas.* Clinical cancer research, 2000. **6**(9): p. 3417-3423.
272. Ohno, N., et al., *Expression of functional lung resistance–related protein predicts poor outcome in adult T-cell leukemia.* Blood, 2001. **98**(4): p. 1160-1165.
273. Fan, K., et al., *Expression of multidrug resistance-related markers in gastric cancer.* Anticancer research, 1999. **20**(6C): p. 4809-4814.
274. Park, J.-G., et al., *Chemosensitivity patterns and expression of human multidrug resistance-associated MDR1 gene by human gastric and colorectal carcinoma cell lines.* Journal of the National Cancer Institute, 1990. **82**(3): p. 193-198.
275. Robey-Cafferty, S.S., M.L. Rutledge, and J.M. Bruner, *Expression of a multidrug resistance gene in esophageal adenocarcinoma: Correlation with response to chemotherapy and comparison with gastric adenocarcinoma.* American journal of clinical pathology, 1990. **93**(1): p. 1-7.
276. Okuyama, T., et al., *Expression of glutathione s-transferasepi and sensitivity of human gastric cancer cells to cisplatin.* Cancer, 1994. **74**(4): p. 1230-1236.
277. Kodera, Y., et al., *Gene expression of 5-fluorouracil metabolic enzymes in primary gastric cancer: correlation with drug sensitivity against 5-fluorouracil.* Cancer letters, 2007. **252**(2): p. 307-313.
278. Gao, J., et al., *Thymidine Phosphorylase/β-tubulin III expressions predict the response in Chinese advanced gastric cancer patients receiving first-line capecitabine plus paclitaxel.* BMC cancer, 2011. **11**(1): p. 1.

279. Hua, D., et al., *Thymidylate synthase and thymidine phosphorylase gene expression as predictive parameters for the efficacy of 5-fluorouraci-based adjuvant chemotherapy for gastric cancer.* World journal of gastroenterology, 2007. **13**(37): p. 5030.
280. Son, Y.S., et al., *Reduced activity of topoisomerase II in an Adriamycin-resistant human stomach-adenocarcinoma cell line.* Cancer chemotherapy and pharmacology, 1998. **41**(5): p. 353-360.
281. Geng, M., L. Wang, and P. Li, *Correlation between chemosensitivity to anticancer drugs and Bcl-2 expression in gastric cancer.* Int J Clin Exp Pathol, 2013. **6**(11): p. 2554-2559.
282. Li, Y., et al., *Tumor chemosensitivity is correlated with expression of multidrug resistance associated factors in variously differentiated gastric carcinoma tissues.* Hepato-gastroenterology, 2012. **60**(121): p. 213-216.
283. Yu, D.-f., et al., *Bcl-2 gene silence enhances the sensitivity toward 5-Fluorouracil in gastric adenocarcinoma cells.* Biomedicine & Pharmacotherapy, 2013. **67**(7): p. 615-619.
284. Kim, C.W., et al., *p53 restoration can overcome cisplatin resistance through inhibition of Akt as well as induction of Bax.* International journal of oncology, 2013. **43**(5): p. 1495-1502.
285. Suh, D.H., et al., *Metabolic approaches to overcoming chemoresistance in ovarian cancer.* Annals of the New York Academy of Sciences, 2011. **1229**(1): p. 53-60.
286. Oki, E., et al., *Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer.* International journal of cancer, 2005. **117**(3): p. 376-380.
287. Murakami, D., et al., *Expression of phosphorylated Akt (pAkt) in gastric carcinoma predicts prognosis and efficacy of chemotherapy.* Gastric Cancer, 2007. **10**(1): p. 45-51.
288. KIM, S.H., Y.S. JUHNN, and Y.S. SONG, *Akt involvement in paclitaxel chemoresistance of human ovarian cancer cells.* Annals of the New York Academy of Sciences, 2007. **1095**(1): p. 82-89.
289. Guo, X., et al., *Increased p38-MAPK is responsible for chemotherapy resistance in human gastric cancer cells.* BMC cancer, 2008. **8**(1): p. 1.
290. Nakamura, J., et al., *Hypoxia-inducible factor-1alpha expression predicts the response to 5-fluorouracil-based adjuvant chemotherapy in advanced gastric cancer.* Oncol Rep, 2009. **22**(4): p. 693-699.
291. Rohwer, N., et al., *Hypoxia-inducible factor 1α determines gastric cancer chemosensitivity via modulation of p53 and NF-κB.* PloS one, 2010. **5**(8): p. e12038.
292. Sun, L., et al., *Gastric cancer cell adhesion to laminin enhances acquired chemotherapeutic drug resistance mediated by MGr1-Ag/37LRP.* Oncology reports, 2014. **32**(1): p. 105-114.
293. Sun, L., et al., *MGr1-Ag/37LRP induces cell adhesion-mediated drug resistance through FAK/PI3K and MAPK pathway in gastric cancer.* Cancer science, 2014. **105**(6): p. 651-659.
294. Yang, Z., et al., *Acquisition of resistance to trastuzumab in gastric cancer cells is associated with activation of IL-6/STAT3/Jagged-1/Notch positive feedback loop.* Oncotarget, 2015. **6**(7): p. 5072-5087.

295. Kwon, O.-H., et al., *TWEAK/Fn14 signaling mediates gastric cancer cell resistance to 5-fluorouracil via NF-κB activation.* International journal of oncology, 2014. **44**(2): p. 583-590.
296. Mallini, P., et al., *Epithelial-to-mesenchymal transition: what is the impact on breast cancer stem cells and drug resistance.* Cancer treatment reviews, 2014. **40**(3): p. 341-348.
297. Xiang, J., et al., *Grhl2 reduces invasion and migration through inhibition of TGFβ-induced EMT in gastric cancer.* Oncogenesis, 2017. **6**(1): p. e284.
298. Findlay, V.J., et al., *Epithelial-to-mesenchymal transition and the cancer stem cell phenotype: insights from cancer biology with therapeutic implications for colorectal cancer.* Cancer gene therapy, 2014. **21**(5): p. 181-187.
299. Gupta, N., et al., *Notch3 induces epithelial–mesenchymal transition and attenuates carboplatin-induced apoptosis in ovarian cancer cells.* Gynecologic oncology, 2013. **130**(1): p. 200-206.
300. Zhao, J., et al., *Prognostic significance of the epithelial-to-mesenchymal transition markers e-cadherin, vimentin and twist in bladder cancer.* International braz j urol, 2014. **40**(2): p. 179-189.
301. Li, Y., et al., *Regulation of EMT by Notch signaling pathway in tumor progression.* Current cancer drug targets, 2013. **13**(9): p. 957-962.
302. Cufí, S., et al., *Metformin against TGFβ-induced epithelial-to-mesenchymal transition (EMT): from cancer stem cells to aging-associated fibrosis.* Cell cycle, 2010. **9**(22): p. 4461-4468.
303. Li, L., et al., *MDR1/P-gp and VEGF synergistically enhance the invasion of Hep-2 cells with multidrug resistance induced by taxol.* Annals of surgical oncology, 2009. **16**(5): p. 1421-1428.
304. Jang, J.-Y., et al., *Adenovirus adenine nucleotide translocator-2 shRNA effectively induces apoptosis and enhances chemosensitivity by the down-regulation of ABCG2 in breast cancer stem-like cells.* Experimental & molecular medicine, 2012. **44**(4): p. 251-259.
305. Saxena, M., et al., *Transcription factors that mediate epithelial–mesenchymal transition lead to multidrug resistance by upregulating ABC transporters.* Cell death & disease, 2011. **2**(7): p. e179.
306. Borovski, T., et al., *Cancer stem cell niche: the place to be.* Cancer research, 2011. **71**(3): p. 634-639.
307. Eaves, C.J., *Cancer stem cells: Here, there, everywhere?* Nature, 2008. **456**(7222): p. 581-582.
308. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proceedings of the National Academy of Sciences, 2003. **100**(7): p. 3983-3988.
309. Bekaii-Saab, T. and B. El-Rayes, *Identifying and targeting cancer stem cells in the treatment of gastric cancer.* Cancer, 2017.
310. Kakar, S.S. and M.Z. Ratajczak, *Targeting cancer stem cells in ovarian cancer*. 2016, AACR.
311. Zeuner, A., et al., *Colorectal cancer stem cells: from the crypt to the clinic.* Cell stem cell, 2014. **15**(6): p. 692-705.
312. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* nature, 2001. **414**(6859): p. 105-111.

313. Polyak, K. and R.A. Weinberg, *Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits.* Nature Reviews Cancer, 2009. **9**(4): p. 265-273.
314. Dean, M., T. Fojo, and S. Bates, *Tumour stem cells and drug resistance.* Nature Reviews Cancer, 2005. **5**(4): p. 275-284.
315. Wu, K.-J., *Direct activation of Bmi1 by Twist1: implications in cancer stemness, epithelial-mesenchymal transition, and clinical significance.* Chang Gung Med J, 2011. **34**(3): p. 229-238.
316. Breitman, T., S.E. Selonick, and S.J. Collins, *Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid.* Proceedings of the National Academy of Sciences, 1980. **77**(5): p. 2936-2940.
317. Breitman, T., S.J. Collins, and B. Keene, *Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid.* Blood, 1981. **57**(6): p. 1000-1004.
318. Kawamata, H., et al., *Differentiation-inducing therapy for solid tumors.* Current pharmaceutical design, 2006. **12**(3): p. 379-385.
319. Vescovi, A.L., R. Galli, and B.A. Reynolds, *Brain tumour stem cells.* Nature Reviews Cancer, 2006. **6**(6): p. 425-436.
320. Vargo-Gogola, T. and J.M. Rosen, *Modelling breast cancer: one size does not fit all.* Nature Reviews Cancer, 2007. **7**(9): p. 659-672.
321. Takaishi, S., et al., *Identification of gastric cancer stem cells using the cell surface marker CD44.* Stem cells, 2009. **27**(5): p. 1006-1020.
322. Lu, X.-X., et al., *Expression and clinical significance of CD73 and hypoxia-inducible factor-1alpha in gastric carcinoma.* World J Gastroenterol, 2013. **19**(12): p. 1912-1918.
323. Jiang, J., et al., *Trastuzumab (herceptin) targets gastric cancer stem cells characterized by CD90 phenotype.* Oncogene, 2012. **31**(6): p. 671-682.
324. Mărgăritescu, C., et al., *The immunohistochemical profile of the adenocarcinoma of upper gastric pole.* Rom J Morphol Embryol, 2007. **48**(3): p. 215-235.
325. Hasegawa, T., et al., *Gastrointestinal stromal tumor: consistent CD117 immunostaining for diagnosis, and prognostic classification based on tumor size and MIB-1 grade.* Human pathology, 2002. **33**(6): p. 669-676.
326. Sahlberg, S.H., et al., *Evaluation of cancer stem cell markers CD133, CD44, CD24: association with AKT isoforms and radiation resistance in colon cancer cells.* PloS one, 2014. **9**(4): p. e94621.
327. Yasumoto, K., et al., *Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer.* Cancer research, 2006. **66**(4): p. 2181-2187.
328. Zhao, X., F. Wang, and M. Hou, *Expression of stem cell markers nanog and PSCA in gastric cancer and its significance.* Oncology letters, 2016. **11**(1): p. 442-448.
329. Chen, Z., et al., *Oct4, a novel marker for human gastric cancer.* Journal of surgical oncology, 2009. **99**(7): p. 414-419.
330. Matsuoka, J., et al., *Role of the stemness factors sox2, oct3/4, and nanog in gastric carcinoma.* Journal of Surgical Research, 2012. **174**(1): p. 130-135.

331. Yang, J., et al., *Clinical significance of the expression of DNA methyltransferase proteins in gastric cancer.* Mol Med Rep, 2011. **4**(6): p. 1139-1143.
332. Gulotta, M., et al., *Effects of cell volume regulating osmolytes on glycerol-3-phosphate binding to triosephosphate isomerase.* Biochemistry, 2007. **46**(35): p. 10055.
333. Baud, V. and M. Karin, *Is NF-κB a good target for cancer therapy? Hopes and pitfalls.* Nature reviews Drug discovery, 2009. **8**(1): p. 33-40.
334. Burger, J. and A. Peled, *CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers.* Leukemia, 2009. **23**(1): p. 43-52.
335. Chen, A.M., et al., *Co-delivery of doxorubicin and Bcl-2 siRNA by mesoporous silica nanoparticles enhances the efficacy of chemotherapy in multidrug-resistant cancer cells.* Small, 2009. **5**(23): p. 2673-2677.
336. Guo, G., et al., *Oncogenic E17K mutation in the pleckstrin homology domain of AKT1 promotes v-Abl-mediated pre-B-cell transformation and survival of Pim-deficient cells.* Oncogene, 2010. **29**(26): p. 3845-3853.
337. Harrison, H., et al., *Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor.* Cancer research, 2010. **70**(2): p. 709-718.
338. Roy, S.K., R.K. Srivastava, and S. Shankar, *Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer.* Journal of molecular signaling, 2010. **5**(1): p. 10.
339. Murphy, M. and B. Stordal, *Erlotinib or gefitinib for the treatment of relapsed platinum pretreated non-small cell lung cancer and ovarian cancer: a systematic review.* Drug Resistance Updates, 2011. **14**(3): p. 177-190.
340. Lee, C.H., et al., *NF-κB as a potential molecular target for cancer therapy.* Biofactors, 2007. **29**(1): p. 19-35.
341. Pradhan, A., Q.T. Lambert, and G.W. Reuther, *Transformation of hematopoietic cells and activation of JAK2-V617F by IL-27R, a component of a heterodimeric type I cytokine receptor.* Proceedings of the National Academy of Sciences, 2007. **104**(47): p. 18502-18507.
342. Shin, K., et al., *Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder.* Nature, 2011. **472**(7341): p. 110-114.
343. Katoh, M., *Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells.* Current pharmaceutical biotechnology, 2011. **12**(2): p. 160-170.
344. Wong, V.W., et al., *Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling.* Nature cell biology, 2012. **14**(4): p. 401-408.
345. Karhadkar, S.S., et al., *Hedgehog signalling in prostate regeneration, neoplasia and metastasis.* Nature, 2004. **431**(7009): p. 707-712.
346. Zhao, C., et al., *Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia.* Nature, 2009. **458**(7239): p. 776-779.
347. Acevedo, V.D., et al., *Inducible FGFR-1 activation leads to irreversible prostate adenocarcinoma and an epithelial-to-mesenchymal transition.* Cancer cell, 2007. **12**(6): p. 559-571.

348. Lu, Z., et al., *Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of β-catenin, and enhanced tumor cell invasion.* Cancer cell, 2003. **4**(6): p. 499-515.
349. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells.* Breast Cancer Research, 2004. **6**(6): p. R605.
350. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs.* Science, 2001. **294**(5543): p. 853-858.
351. Behm-Ansmant, I., et al., *mRNA degradation by miRNAs and GW182 requires both CCR4: NOT deadenylase and DCP1: DCP2 decapping complexes.* Genes & development, 2006. **20**(14): p. 1885-1898.
352. Giraldez, A.J., et al., *Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs.* science, 2006. **312**(5770): p. 75-79.
353. Wu, L., J. Fan, and J.G. Belasco, *MicroRNAs direct rapid deadenylation of mRNA.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(11): p. 4034-4039.
354. Kozomara, A. and S. Griffiths-Jones, *miRBase: annotating high confidence microRNAs using deep sequencing data.* Nucleic acids research, 2014. **42**(D1): p. D68-D73.
355. Dalmay, T., *Mechanism of miRNA-mediated repression of mRNA translation.* Essays in biochemistry, 2013. **54**: p. 29-38.
356. Croce, C.M., *Causes and consequences of microRNA dysregulation in cancer.* Nature reviews genetics, 2009. **10**(10): p. 704-714.
357. Volinia, S., et al., *A microRNA expression signature of human solid tumors defines cancer gene targets.* Proceedings of the National academy of Sciences of the United States of America, 2006. **103**(7): p. 2257-2261.
358. Liu, C. and D.G. Tang, *MicroRNA regulation of cancer stem cells.* Cancer research, 2011. **71**(18): p. 5950-5954.
359. Katoh, Y. and M. Katoh, *Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review).* International journal of molecular medicine, 2008. **22**(3): p. 271.
360. Gregory, P.A., et al., *MicroRNAs as regulators of epithelial-mesenchymal transition.* Cell cycle, 2008. **7**(20): p. 3112-3117.
361. Schetter, A.J., et al., *MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma.* Jama, 2008. **299**(4): p. 425-436.
362. Tong, A. and J. Nemunaitis, *Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy?* Cancer gene therapy, 2008. **15**(6): p. 341-355.
363. Tryndyak, V.P., F.A. Beland, and I.P. Pogribny, *E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells.* International journal of cancer, 2010. **126**(11): p. 2575-2583.
364. Wiklund, E.D., et al., *Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer.* International journal of cancer, 2011. **128**(6): p. 1327-1334.
365. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.* Nature cell biology, 2008. **10**(5): p. 593-601.

366. Valladares-Ayerbes, M., et al., *Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer.* Journal of translational medicine, 2012. **10**(1): p. 186.
367. Schliekelman, M.J., et al., *Targets of the tumor suppressor miR-200 in regulation of the epithelial–mesenchymal transition in cancer.* Cancer research, 2011. **71**(24): p. 7670-7682.
368. Jiang, J., et al., *Real-time expression profiling of microRNA precursors in human cancer cell lines.* Nucleic acids research, 2005. **33**(17): p. 5394-5403.
369. Gottardo, F., et al. *Micro-RNA profiling in kidney and bladder cancers*. in *Urologic Oncology: Seminars and Original Investigations*. 2007. Elsevier.
370. Ryan, D.G., M. Oliveira-Fernandes, and R.M. Lavker, *MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity.* Mol Vis, 2006. **12**(1175): p. 84.
371. Chan, J.A., A.M. Krichevsky, and K.S. Kosik, *MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells.* Cancer research, 2005. **65**(14): p. 6029-6033.
372. Iorio, M.V., et al., *MicroRNA gene expression deregulation in human breast cancer.* Cancer research, 2005. **65**(16): p. 7065-7070.
373. Si, M., et al., *miR-21-mediated tumor growth.* Oncogene, 2007. **26**(19): p. 2799-2803.
374. Zhang, Z., et al., *miR-21 plays a pivotal role in gastric cancer pathogenesis and progression.* Laboratory investigation, 2008. **88**(12): p. 1358-1366.
375. Ciafre, S., et al., *Extensive modulation of a set of microRNAs in primary glioblastoma.* Biochemical and biophysical research communications, 2005. **334**(4): p. 1351-1358.
376. Zhu, S., et al., *MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1).* Journal of Biological Chemistry, 2007. **282**(19): p. 14328-14336.
377. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.* Proceedings of the National academy of Sciences of the United States of America, 2004. **101**(9): p. 2999-3004.
378. Minna, J.D., J.A. Roth, and A.F. Gazdar, *Focus on lung cancer.* Cancer cell, 2002. **1**(1): p. 49-52.
379. Yanaihara, N., et al., *Unique microRNA molecular profiles in lung cancer diagnosis and prognosis.* Cancer cell, 2006. **9**(3): p. 189-198.
380. Ohshima, K., et al., *Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line.* PloS one, 2010. **5**(10): p. e13247.
381. Singh, A. and J. Settleman, *EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer.* Oncogene, 2010. **29**(34): p. 4741-4751.
382. Xu, J., S. Lamouille, and R. Derynck, *TGF-β-induced epithelial to mesenchymal transition.* Cell research, 2009. **19**(2): p. 156-172.
383. Mani, S.A., et al., *The epithelial-mesenchymal transition generates cells with properties of stem cells.* Cell, 2008. **133**(4): p. 704-715.

384. Zhi, Y., et al., *B7H1 expression and epithelial-to-mesenchymal transition phenotypes on colorectal cancer stem-like cells.* PloS one, 2015. **10**(8): p. e0135528.
385. Garg, M., *Urothelial cancer stem cells and epithelial plasticity: current concepts and therapeutic implications in bladder cancer.* Cancer and Metastasis Reviews, 2015. **34**(4): p. 691-701.
386. Chang, Y.-W., et al., *Diverse targets of β-catenin during the epithelial–mesenchymal transition define cancer stem cells and predict disease relapse.* Cancer research, 2015. **75**(16): p. 3398-3410.
387. Radisky, D.C. and M.A. LaBarge, *Epithelial-mesenchymal transition and the stem cell phenotype.* Cell stem cell, 2008. **2**(6): p. 511-512.
388. Scheel, C. and R.A. Weinberg, *Phenotypic plasticity and epithelial-mesenchymal transitions in cancer and normal stem cells?* International journal of cancer, 2011. **129**(10): p. 2310-2314.
389. Singh, A., et al., *A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cell survival.* Cancer cell, 2009. **15**(6): p. 489-500.
390. Wang, Z., et al., *Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway.* Cancer research, 2009. **69**(6): p. 2400-2407.
391. Sarkar, F.H., et al., *Pancreatic cancer stem cells and EMT in drug resistance and metastasis.* Minerva chirurgica, 2009. **64**(5): p. 489.
392. Wang, H., et al., *Transforming growth factor β-induced epithelial-mesenchymal transition increases cancer stem-like cells in the PANC-1 cell line.* Oncology letters, 2012. **3**(1): p. 229-233.
393. Wang, S.-s., et al., *Links between cancer stem cells and epithelial–mesenchymal transition.* OncoTargets and therapy, 2015. **8**: p. 2973.
394. Scharenberg, C.W., M.A. Harkey, and B. Torok-Storb, *The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors.* Blood, 2002. **99**(2): p. 507-512.
395. Kim, M., et al., *The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells.* Clinical Cancer Research, 2002. **8**(1): p. 22-28.
396. Schinkel, A., et al., *Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs.* Cell, 1994. **77**(4): p. 491-502.
397. Dean, M., T. Fojo, and S. Bates, *TUMOUR STEM CELLS AND DRUG RESISTANCE.* NATURE REVIEWS| CANCER, 2005. **5**: p. 275.
398. Dean, M., *ABC transporters, drug resistance, and cancer stem cells.* Journal of mammary gland biology and neoplasia, 2009. **14**(1): p. 3-9.
399. Chartrain, M., et al., *Melanoma chemotherapy leads to the selection of ABCB5-expressing cells.* PloS one, 2012. **7**(5): p. e36762.
400. Surowiak, P., et al., *ABCC2 (MRP2, cMOAT) can be localized in the nuclear membrane of ovarian carcinomas and correlates with resistance to cisplatin and clinical outcome.* Clinical Cancer Research, 2006. **12**(23): p. 7149-7158.

401. Vasilyeva, A., et al., *Hepatocellular shuttling and recirculation of sorafenib-glucuronide is dependent on Abcc2, Abcc3, and Oatp1a/1b.* Cancer research, 2015. **75**(13): p. 2729-2736.
402. Lagas, J.S., et al., *Multidrug resistance protein 2 is an important determinant of paclitaxel pharmacokinetics.* Clinical Cancer Research, 2006. **12**(20): p. 6125-6132.
403. Campa, D., et al., *A comprehensive study of polymorphisms in ABCB1, ABCC2 and ABCG2 and lung cancer chemotherapy response and prognosis.* International journal of cancer, 2012. **131**(12): p. 2920-2928.
404. Simon, N., et al., *Impact of ABCC2 polymorphisms on high-dose methotrexate pharmacokinetics in patients with lymphoid malignancy.* The pharmacogenomics journal, 2013. **13**(6): p. 507-513.
405. Visvader, J.E. and G.J. Lindeman, *Cancer stem cells in solid tumours: accumulating evidence and unresolved questions.* Nature reviews cancer, 2008. **8**(10): p. 755-768.
406. Folkins, C., et al., *Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors.* Cancer research, 2007. **67**(8): p. 3560-3564.
407. Williams, R., et al., *Maturation of tumor vasculature by interferon-β disrupts the vascular niche of glioma stem cells.* Anticancer research, 2010. **30**(9): p. 3301-3308.
408. Chen, K., Y.-h. Huang, and J.-l. Chen, *Understanding and targeting cancer stem cells: therapeutic implications and challenges.* Acta Pharmacologica Sinica, 2013. **34**(6): p. 732-740.
409. Merchant, A.A. and W. Matsui, *Targeting Hedgehog—a cancer stem cell pathway.* Clinical cancer research, 2010. **16**(12): p. 3130-3140.
410. Jin, L., et al., *Targeting of CD44 eradicates human acute myeloid leukemic stem cells.* Nature medicine, 2006. **12**(10): p. 1167-1174.
411. Stagg, J., et al., *Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis.* Proceedings of the National Academy of Sciences, 2010. **107**(4): p. 1547-1552.
412. Yang, Z.F., et al., *Significance of CD90+ cancer stem cells in human liver cancer.* Cancer cell, 2008. **13**(2): p. 153-166.
413. Fonsatti, E., et al., *Targeting cancer vasculature via endoglin/CD105: a novel antibody-based diagnostic and therapeutic strategy in solid tumours.* Cardiovascular research, 2010. **86**(1): p. 12-19.
414. Zhang, S., et al., *Identification and characterization of ovarian cancer-initiating cells from primary human tumors.* Cancer research, 2008. **68**(11): p. 4311-4320.
415. Liu, G., et al., *Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma.* Molecular cancer, 2006. **5**(1): p. 67.
416. Warrell Jr, R.P., et al., *Acute promyelocytic leukemia.* New England Journal of Medicine, 1993. **329**(3): p. 177-189.
417. Olsson, I., et al., *Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1α, 25-dihydroxycholecalciferol.* Cancer research, 1983. **43**(12 Part 1): p. 5862-5867.
418. Arcangeli, A., et al., *Polar/apolar compounds induce leukemia cell differentiation by modulating cell-surface potential.* Proceedings of the National Academy of Sciences, 1993. **90**(12): p. 5858-5862.

419. Xu, Q., et al., *Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens.* Stem cells, 2009. **27**(8): p. 1734-1740.
420. Yingling, J.M., K.L. Blanchard, and J.S. Sawyer, *Development of TGF-β signalling inhibitors for cancer therapy.* Nature reviews Drug discovery, 2004. **3**(12): p. 1011-1022.
421. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy.* Nature Reviews Cancer, 2012. **12**(4): p. 252-264.
422. Wouters, B.G. and M. Koritzinsky, *Hypoxia signalling through mTOR and the unfolded protein response in cancer.* Nature Reviews Cancer, 2008. **8**(11): p. 851-864.
423. Ginestier, C., et al., *Mevalonate metabolism regulates Basal breast cancer stem cells and is a potential therapeutic target.* Stem cells, 2012. **30**(7): p. 1327-1337.
424. Massard, C., E. Deutsch, and J. Soria, *Tumour stem cell-targeted treatment: elimination or differentiation.* Annals of oncology, 2006. **17**(11): p. 1620-1624.
425. Matushansky, I., *Solid tumor differentiation therapy–is it possible?* Oncotarget, 2012. **3**(5): p. 559-567.
426. Pierce, G. and E. Verney, *An in vitro and in vivo study of differentiation in teratocarcinomas.* Cancer, 1961. **14**(5): p. 1017-1029.
427. Nowak, D., D. Stewart, and H.P. Koeffler, *Differentiation therapy of leukemia: 3 decades of development.* Blood, 2009. **113**(16): p. 3655-3665.
428. Huang, M., et al., *Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia.* Blood, 1988. **72**(2): p. 567-572.
429. Warrell Jr, R.P., et al., *Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid).* New England Journal of Medicine, 1991. **324**(20): p. 1385-1393.
430. Yan, M., et al., *IKKα restoration via EZH2 suppression induces nasopharyngeal carcinoma differentiation.* Nature communications, 2014. **5**.
431. Spira, A.I. and M.A. Carducci, *Differentiation therapy.* Current opinion in pharmacology, 2003. **3**(4): p. 338-343.
432. Sell, S., *Stem cell origin of cancer and differentiation therapy.* Critical reviews in oncology/hematology, 2004. **51**(1): p. 1-28.
433. Tubiana, M. and E. Malaise, *[Cell proliferation kinetics of tumors and cancer treatment].* Pathologie-biologie, 1973. **21**(6): p. 647-664.
434. Vreys, V. and G. David, *Mammalian heparanase: what is the message?* Journal of cellular and molecular medicine, 2007. **11**(3): p. 427-452.
435. Shteingauz, A., N. Ilan, and I. Vlodavsky, *Processing of heparanase is mediated by syndecan-1 cytoplasmic domain and involves syntenin and α-actinin.* Cellular and molecular life sciences, 2014. **71**(22): p. 4457-4470.
436. Zetser, A., et al., *Processing and activation of latent heparanase occurs in lysosomes.* Journal of cell science, 2004. **117**(11): p. 2249-2258.
437. Nobuhisa, T., et al., *Emergence of nuclear heparanase induces differentiation of human mammary cancer cells.* Biochemical and biophysical research communications, 2005. **331**(1): p. 175-180.
438. Nobuhisa, T., et al., *Translocation of heparanase into nucleus results in cell differentiation.* Cancer science, 2007. **98**(4): p. 535-540.

439. Adachi, Y., et al., *Pathology and prognosis of gastric carcinoma.* Cancer, 2000. **89**(7): p. 1418-1424.
440. Rohloff, J., et al., *Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma.* British journal of cancer, 2002. **86**(8): p. 1270-1275.
441. Parish, C.R., C. Freeman, and M.D. Hulett, *Heparanase: a key enzyme involved in cell invasion.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2001. **1471**(3): p. M99-M108.
442. Marchetti, D. and G.L. Nicolson, *Human heparanase: a molecular determinant of brain metastasis.* Advances in enzyme regulation, 2001. **41**(1): p. 343-359.
443. Vlodavsky, I., et al., *Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis.* Nature medicine, 1999. **5**(7): p. 793.
444. Sanderson, R.D., et al., *Heparan sulfate proteoglycans and heparanase—partners in osteolytic tumor growth and metastasis.* Matrix Biology, 2004. **23**(6): p. 341-352.
445. Nakajima, M., T. Irimura, and G.L. Nicolson, *Tumor metastasis-associated heparanase (heparan sulfate endoglycosidase) activity in human melanoma cells.* Cancer letters, 1986. **31**(3): p. 277-283.
446. Quiros, R.M., et al., *Elevated serum heparanase-1 levels in patients with pancreatic carcinoma are associated with poor survival.* Cancer, 2006. **106**(3): p. 532-540.
447. Cohen, I., et al., *Heparanase promotes growth, angiogenesis and survival of primary breast tumors.* International journal of cancer, 2006. **118**(7): p. 1609-1617.
448. Beckhove, P., et al., *Heparanase expression at the invasion front of human head and neck cancers and correlation with poor prognosis.* Clinical cancer research, 2005. **11**(8): p. 2899-2906.
449. YOSHIONAOMOTO, M., et al., *The role of heparanase in gastrointestinal cancer (Review).* Oncology reports, 2005. **14**: p. 3-8.
450. Vlodavsky, I., et al. *Mammalian heparanase: involvement in cancer metastasis, angiogenesis and normal development*. in *Seminars in cancer biology*. 2002. Elsevier.
451. Maxhimer, J.B., et al., *Heparanase-1 expression is associated with the metastatic potential of breast cancer.* Surgery, 2002. **132**(2): p. 326-333.
452. Watanabe, M., et al., *Heparanase expression and angiogenesis in endometrial cancer.* Gynecologic and obstetric investigation, 2003. **56**(2): p. 77-82.
453. Joyce, J.A., et al., *A functional heparan sulfate mimetic implicates both heparanase and heparan sulfate in tumor angiogenesis and invasion in a mouse model of multistage cancer.* Oncogene, 2005. **24**(25): p. 4037-4051.
454. Davidson, B., et al., *Heparanase expression correlates with poor survival in metastatic ovarian carcinoma.* Gynecologic oncology, 2007. **104**(2): p. 311-319.
455. Takahashi, H., et al., *Clinical significance of heparanase activity in primary resected non-small cell lung cancer.* Lung Cancer, 2004. **45**(2): p. 207-214.

456. Gohji, K., et al., *Expression of three extracellular matrix degradative enzymes in bladder cancer.* International journal of cancer, 2001. **95**(5): p. 295-301.
457. Nakajima, M., et al., *Heparan sulfate degradation: relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines.* Science, 1983. **220**(4597): p. 611-613.
458. Vlodavsky, I., et al., *Lymphoma cell-mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relationship to tumor cell metastasis.* Cancer Research, 1983. **43**(6): p. 2704-2711.
459. Edovitsky, E., et al., *Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis.* Journal of the National Cancer Institute, 2004. **96**(16): p. 1219-1230.
460. Lerner, I., et al., *Function of heparanase in prostate tumorigenesis: potential for therapy.* Clinical Cancer Research, 2008. **14**(3): p. 668-676.
461. Folkman, J., et al., *A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane.* The American journal of pathology, 1988. **130**(2): p. 393.
462. Vlodavsky, I., et al., *Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix.* Proceedings of the National Academy of Sciences, 1987. **84**(8): p. 2292-2296.
463. Elkin, M., et al., *Heparanase as mediator of angiogenesis: mode of action.* The FASEB Journal, 2001. **15**(9): p. 1661-1663.
464. Friedmann, Y., et al., *Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma: evidence for its role in colonic tumorigenesis.* The American journal of pathology, 2000. **157**(4): p. 1167-1175.
465. Vlodavsky, I., et al., *Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor.* Cancer and Metastasis Reviews, 1996. **15**(2): p. 177-186.
466. Kelly, T., et al., *High heparanase activity in multiple myeloma is associated with elevated microvessel density.* Cancer research, 2003. **63**(24): p. 8749-8756.
467. Sanderson, R.D. and Y. Yang, *Syndecan-1: a dynamic regulator of the myeloma microenvironment.* Clinical & experimental metastasis, 2008. **25**(2): p. 149-159.
468. Seidel, C., et al., *Serum syndecan-1: a new independent prognostic marker in multiple myeloma.* Blood, 2000. **95**(2): p. 388-392.
469. Purushothaman, A., et al., *Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis.* Blood, 2010. **115**(12): p. 2449-2457.
470. Yang, Y., et al., *Soluble syndecan-1 promotes growth of myeloma tumors in vivo.* Blood, 2002. **100**(2): p. 610-617.
471. Mahtouk, K., et al., *Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma.* Blood, 2007. **109**(11): p. 4914-4923.

472. Yang, Y., et al., *Heparanase Enhances Syndecan-1 Shedding A NOVEL MECHANISM FOR STIMULATION OF TUMOR GROWTH AND METASTASIS.* Journal of Biological Chemistry, 2007. **282**(18): p. 13326-13333.
473. Xu, X., et al., *Inverse correlation between heparan sulfate composition and heparanase-1 gene expression in thyroid papillary carcinomas.* Clinical cancer research, 2003. **9**(16): p. 5968-5979.
474. El-Assal, O.N., et al., *The clinicopathological significance of heparanase and basic fibroblast growth factor expressions in hepatocellular carcinoma.* Clinical Cancer Research, 2001. **7**(5): p. 1299-1305.
475. Kim, A.W., et al., *Human heparanase-1 gene expression in pancreatic adenocarcinoma.* Journal of gastrointestinal surgery, 2002. **6**(2): p. 167-172.
476. Gohji, K., et al., *Heparanase protein and gene expression in bladder cancer.* The Journal of urology, 2001. **166**(4): p. 1286-1290.
477. Shinyo, Y., et al., *Heparanase expression is an independent prognostic factor in patients with invasive cervical cancer.* Annals of oncology, 2003. **14**(10): p. 1505-1510.
478. Takaoka, M., et al., *Heparanase expression correlates with invasion and poor prognosis in gastric cancers.* Laboratory investigation, 2003. **83**(5): p. 613-622.
479. Tang, W., et al., *Heparanase: a key enzyme in invasion and metastasis of gastric carcinoma.* Modern pathology, 2002. **15**(6): p. 593-598.
480. Ogishima, T., et al., *Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer.* Clinical Cancer Research, 2005. **11**(3): p. 1028-1036.
481. Mikami, S., et al., *Loss of Syndecan-1 and Increased Expression of Heparanase in Invasive Esophageal Carcinomas.* Japanese journal of cancer research, 2001. **92**(10): p. 1062-1073.
482. Bitan, M., et al., *Heparanase expression in human leukemias is restricted to acute myeloid leukemias.* Experimental hematology, 2002. **30**(1): p. 34-41.
483. Sato, T., et al., *Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis.* Journal of surgical oncology, 2004. **87**(4): p. 174-181.
484. Gingis-Velitski, S., et al., *Heparanase induces endothelial cell migration via protein kinase B/Akt activation.* Journal of Biological Chemistry, 2004. **279**(22): p. 23536-23541.
485. Doviner, V., et al., *Spatial and temporal heparanase expression in colon mucosa throughout the adenoma-carcinoma sequence.* Modern pathology, 2006. **19**(6): p. 878-888.
486. Zetser, A., et al., *Heparanase affects adhesive and tumorigenic potential of human glioma cells.* Cancer Research, 2003. **63**(22): p. 7733-7741.
487. Cohen-Kaplan, V., et al., *Heparanase induces VEGF C and facilitates tumor lymphangiogenesis.* International journal of cancer, 2008. **123**(11): p. 2566-2573.
488. Alitalo, K. and P. Carmeliet, *Molecular mechanisms of lymphangiogenesis in health and disease.* Cancer cell, 2002. **1**(3): p. 219-227.

489. Cohen-Kaplan, V., et al., *Heparanase augments epidermal growth factor receptor phosphorylation: correlation with head and neck tumor progression.* Cancer Research, 2008. **68**(24): p. 10077-10085.
490. Nadir, Y., et al., *Hemostatic balance on the surface of leukemic cells: the role of tissue factor and urokinase plasminogen activator receptor.* Haematologica, 2005. **90**(11): p. 1549-1556.
491. De Cicco, M., *The prothrombotic state in cancer: pathogenic mechanisms.* Critical reviews in oncology/hematology, 2004. **50**(3): p. 187-196.
492. Nadir, Y., et al., *Heparanase induces tissue factor expression in vascular endothelial and cancer cells.* Journal of Thrombosis and Haemostasis, 2006. **4**(11): p. 2443-2451.
493. Nadir, Y., et al., *Heparanase enhances the generation of activated factor X in the presence of tissue factor and activated factor VII.* Haematologica, 2010. **95**(11): p. 1927-1934.
494. Nadir, Y., et al., *An assay to evaluate heparanase procoagulant activity.* Thrombosis research, 2011. **128**(4): p. e3-e8.
495. Peled, E., et al., *Increased heparanase level and procoagulant activity in orthopedic surgery patients receiving prophylactic dose of enoxaparin.* Thrombosis research, 2012. **130**(1): p. 129-134.
496. Matan, M., et al., *Heparanase procoagulant activity is elevated in women using oral contraceptives.* Human Reproduction, 2013. **28**(9): p. 2372-2380.
497. Kato, H., *Regulation of functions of vascular wall cells by tissue factor pathway inhibitor.* Arteriosclerosis, thrombosis, and vascular biology, 2002. **22**(4): p. 539-548.
498. Lwaleed, B.A. and P.S. Bass, *Tissue factor pathway inhibitor: structure, biology and involvement in disease.* The Journal of pathology, 2006. **208**(3): p. 327-339.
499. Nadir, Y., et al., *Heparanase induces tissue factor pathway inhibitor expression and extracellular accumulation in endothelial and tumor cells.* Thrombosis and haemostasis, 2008. **99**(1): p. 133-141.
500. Iversen, N., A.K. Lindahl, and U. Abildgaard, *Elevated TFPI in malignant disease: relation to cancer type and hypercoagulation.* British journal of haematology, 1998. **102**(4): p. 889-895.
501. Iversen, N., A.K. Lindahl, and U. Abildgaard, *Elevated plasma levels of the factor Xa–TFPI complex in cancer patients.* Thrombosis research, 2002. **105**(1): p. 33-36.
502. Shafat, I., et al., *An ELISA method for the detection and quantification of human heparanase.* Biochemical and biophysical research communications, 2006. **341**(4): p. 958-963.
503. Rockey, D.C., P.D. Bell, and J.A. Hill, *Fibrosis—a common pathway to organ injury and failure.* New England Journal of Medicine, 2015. **372**(12): p. 1138-1149.
504. He, W. and C. Dai, *Key fibrogenic signaling.* Current pathobiology reports, 2015. **3**(2): p. 183-192.
505. Masola, V., et al., *Regulation of heparanase by albumin and advanced glycation end products in proximal tubular cells.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2011. **1813**(8): p. 1475-1482.

506. Masola, V., et al., *Heparanase and syndecan-1 interplay orchestrates fibroblast growth factor-2-induced epithelial-mesenchymal transition in renal tubular cells.* Journal of Biological Chemistry, 2012. **287**(2): p. 1478-1488.
507. Masola, V., et al., *Heparanase is a key player in renal fibrosis by regulating TGF-β expression and activity.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2014. **1843**(9): p. 2122-2128.
508. Gil, N., et al., *Heparanase is essential for the development of diabetic nephropathy in mice.* Diabetes, 2012. **61**(1): p. 208-216.
509. Masola, V., et al., *A new mechanism of action of sulodexide in diabetic nephropathy: inhibits heparanase-1 and prevents FGF-2-induced renal epithelial-mesenchymal transition.* Journal of translational medicine, 2012. **10**(1): p. 213.
510. Davids, J.S., et al., *Chronic cyclooxygenase-2 inhibition promotes myofibroblast-associated intestinal fibrosis.* Cancer Prevention Research, 2010. **3**(3): p. 348-358.
511. Ikeguchi, M., Y. Hirooka, and N. Kaibara, *Heparanase gene expression and its correlation with spontaneous apoptosis in hepatocytes of cirrhotic liver and carcinoma.* European Journal of Cancer, 2003. **39**(1): p. 86-90.
512. Xiao, Y., et al., *Heparanase expression in hepatocellular carcinoma and the cirrhotic liver.* Hepatology research, 2003. **26**(3): p. 192-198.
513. Ohayon, O., et al., *Halofuginone upregulates the expression of heparanase in thioacetamide-induced liver fibrosis in rats.* Laboratory Investigation, 2008. **88**(6): p. 627-633.
514. Goldshmidt, O., et al., *Heparanase expression during normal liver development and following partial hepatectomy.* The Journal of pathology, 2004. **203**(1): p. 594-602.
515. Marchetti, D., J. Li, and R. Shen, *Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase.* Cancer research, 2000. **60**(17): p. 4767-4770.
516. ZENG, Y.-h. and D.-c. PAN, *Heparanase and Tumor Metastasis.* Bulletin of Chinese Cancer, 2004. **3**: p. 010.
517. Casu, B., I. Vlodavsky, and R.D. Sanderson, *Non-anticoagulant heparins and inhibition of cancer.* Pathophysiology of haemostasis and thrombosis, 2007. **36**(3-4): p. 195-203.
518. Ferro, V., et al., *Large-scale preparation of the oligosaccharide phosphate fraction of Pichia holstii NRRL Y-2448 phosphomannan for use in the manufacture of PI-88.* Carbohydrate research, 2001. **332**(2): p. 183-189.
519. Parish, C.R., et al., *Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity.* Cancer research, 1999. **59**(14): p. 3433-3441.
520. Cochran, S., et al., *Probing the interactions of phosphosulfomannans with angiogenic growth factors by surface plasmon resonance.* Journal of medicinal chemistry, 2003. **46**(21): p. 4601-4608.
521. Ferro, V., et al. *PI-88 and novel heparan sulfate mimetics inhibit angiogenesis*. in *Seminars in thrombosis and hemostasis*. 2007. Copyright© 2007 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.

522. Dredge, K., et al., *PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models.* British journal of cancer, 2011. **104**(4): p. 635-642.
523. Brennan, T.V., et al., *Heparan sulfate mimetic PG545-mediated antilymphoma effects require TLR9-dependent NK cell activation.* The Journal of clinical investigation, 2016. **126**(1): p. 207.
524. Ramani, V.C., et al., *Targeting heparanase overcomes chemoresistance and diminishes relapse in myeloma.* Oncotarget, 2016. **7**(2): p. 1598.
525. Ritchie, J.P., et al., *SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis.* Clinical Cancer Research, 2011. **17**(6): p. 1382-1393.
526. Zhou, H., et al., *M402, a novel heparan sulfate mimetic, targets multiple pathways implicated in tumor progression and metastasis.* PloS one, 2011. **6**(6): p. e21106.
527. Tayel, A., et al., *Suramin inhibits hepatic tissue damage in hepatocellular carcinoma through deactivation of heparanase enzyme.* European journal of pharmacology, 2014. **728**: p. 151-160.
528. Tayel, A., et al., *Cytotoxic effects of suramin against HepG2 cells through activation of intrinsic apoptotic pathway.* J. BUON, 2014. **19**(4): p. 1048-54.
529. Li, Y., et al., *Suppression of endoplasmic reticulum stress-induced invasion and migration of breast cancer cells through the downregulation of heparanase.* International journal of molecular medicine, 2013. **31**(5): p. 1234-1242.
530. Weissmann, M., et al., *Heparanase-neutralizing antibodies attenuate lymphoma tumor growth and metastasis.* Proceedings of the National Academy of Sciences, 2016. **113**(3): p. 704-709.
531. Zhang, J., et al., *Multiple antigenic polypeptide composed of heparanase B cell epitopes shrinks human hepatocellular carcinoma in mice.* Oncology reports, 2015. **33**(3): p. 1248-1256.
532. Nakajima, M., et al., *Suramin. A potent inhibitor of melanoma heparanase and invasion.* Journal of Biological Chemistry, 1991. **266**(15): p. 9661-9666.
533. Mitsiades, C.S., et al., *Preclinical studies in support of defibrotide for the treatment of multiple myeloma and other neoplasias.* Clinical Cancer Research, 2009. **15**(4): p. 1210-1221.
534. Palumbo, A., et al., *Melphalan, prednisone, thalidomide and defibrotide in relapsed/refractory multiple myeloma: results of a multicenter phase I/II trial.* Haematologica, 2010. **95**(7): p. 1144-1149.
535. Simmons, S.C., et al., *Anti-heparanase aptamers as potential diagnostic and therapeutic agents for oral cancer.* PloS one, 2014. **9**(10): p. e96846.
536. Dong, W., et al., *Gene silencing of heparanase results in suppression of invasion and migration of hepatoma cells.* World journal of surgical oncology, 2014. **12**(1): p. 85.
537. Liu, M., et al., *Evaluation of the antitumor efficacy of RNAi-mediated inhibition of CDC20 and heparanase in an orthotopic liver tumor model.* Cancer Biotherapy and Radiopharmaceuticals, 2015. **30**(6): p. 233-239.

538. Liu, X., et al., *Lentiviral miR30-based RNA interference against heparanase suppresses melanoma metastasis with lower liver and lung toxicity.* Int J Biol Sci, 2013. **9**(6): p. 564-77.
539. Marchetti, D., et al., *Inhibition of heparanase activity and heparanase-induced angiogenesis by suramin analogues.* International journal of cancer, 2003. **104**(2): p. 167-174.
540. Taylor, C.W., et al., *Effects of suramin on in vitro growth of fresh human tumors.* Journal of the National Cancer Institute, 1992. **84**(7): p. 489-494.
541. Lam, E.T., et al., *Phase I trial of non-cytotoxic suramin as a modulator of docetaxel and gemcitabine therapy in previously treated patients with non-small cell lung cancer.* Cancer chemotherapy and pharmacology, 2010. **66**(6): p. 1019-1029.
542. Hutson, P.R., et al., *Renal clearance, tissue distribution, and CA-125 responses in a phase I trial of suramin.* Clinical cancer research, 1998. **4**(6): p. 1429-1436.
543. Sadashiva, M.P., et al., *Anti-cancer activity of novel dibenzo [b, f] azepine tethered isoxazoline derivatives.* BMC chemical biology, 2012. **12**(1): p. 5.
544. Reininger, A., *Function of von Willebrand factor in haemostasis and thrombosis.* Haemophilia, 2008. **14**(s5): p. 11-26.
545. Harrison, P. and E.M. Cramer, *Platelet α-granules.* Blood reviews, 1993. **7**(1): p. 52-62.
546. Mackman, N., R.E. Tilley, and N.S. Key, *Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis.* Arteriosclerosis, thrombosis, and vascular biology, 2007. **27**(8): p. 1687-1693.
547. Al Dieri, R. and C.H. Hemker, *Thrombin generation in whole blood.* British journal of haematology, 2008. **141**(6): p. 895-895.
548. Gailani, D. and T. Renné, *Intrinsic pathway of coagulation and arterial thrombosis.* Arteriosclerosis, thrombosis, and vascular biology, 2007. **27**(12): p. 2507-2513.
549. Lijnen, H., *Elements of the fibrinolytic system.* Annals of the New York Academy of Sciences, 2001. **936**(1): p. 226-236.
550. Cesarman-Maus, G. and K.A. Hajjar, *Molecular mechanisms of fibrinolysis.* British journal of haematology, 2005. **129**(3): p. 307-321.
551. Willemse, J., E. Heylen, and D. Hendriks, *The intrinsic enzymatic activity of procarboxypeptidase U (TAFI) does not significantly influence the fibrinolysis rate: a rebuttal.* Journal of Thrombosis and Haemostasis, 2007. **5**(6): p. 1334-1336.
552. Sandset, P.M., *Tissue factor pathway inhibitor (TFPI)–an update.* Pathophysiology of Haemostasis and Thrombosis, 1996. **26**(Suppl. 4): p. 154-165.
553. Levi, M., M. Schouten, and T. van der Poll. *Sepsis, coagulation, and antithrombin: old lessons and new insights*. in *Seminars in thrombosis and hemostasis*. 2008. © Thieme Medical Publishers.
554. Van de Wouwer, M., D. Collen, and E.M. Conway, *Thrombomodulin-protein C-EPCR system.* Arteriosclerosis, thrombosis, and vascular biology, 2004. **24**(8): p. 1374-1383.
555. Kobayashi, H., et al., *Role of activated protein C in facilitating basement membrane invasion by tumor cells.* Cancer research, 1994. **54**(1): p. 261-267.

556. Gödecke, W., *Phlegmasia alba dolens*. 1844: Typis C. Lindovii.
557. Donati, M.B., *Cancer and thrombosis.* Pathophysiology of Haemostasis and Thrombosis, 1994. **24**(2): p. 128-131.
558. LEVIN, J. and C.L. CONLEY, *Thrombocytosis associated with malignant disease.* Archives of internal medicine, 1964. **114**(4): p. 497.
559. Wojtukiewicz, M.Z., E. Sierko, and W. Kisiel. *The role of hemostatic system inhibitors in malignancy*. in *Seminars in thrombosis and hemostasis*. 2007. New York: Stratton Intercontinental Medical Book Corporation, c1974-.
560. Bick, R.L. *Coagulation abnormalities in malignancy: a review*. in *Seminars in thrombosis and hemostasis*. 1991.
561. Khorana, A.A., et al., *Risk factors for chemotherapy-associated venous thromboembolism in a prospective observational study.* Cancer, 2005. **104**(12): p. 2822-2829.
562. Rickles, F.R. and R.L. Edwards, *Activation of blood coagulation in cancer: Trousseau's syndrome revisited.* Blood, 1983. **62**(1): p. 14-31.
563. Adcock, D.M., et al., *The hemostatic system and malignancy.* Clinical Lymphoma and Myeloma, 2008. **8**(4): p. 230-236.
564. LaRusch, G.A., et al., *Factor XII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis.* Blood, 2010. **115**(24): p. 5111-5120.
565. Pauer, H.-U., et al., *Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo.* THROMBOSIS AND HAEMOSTASIS-STUTTGART-, 2004. **92**: p. 503-508.
566. Rickles, F.R., M. Levine, and R.L. Edwards, *Hemostatic alterations in cancer patients.* Cancer and Metastasis Reviews, 1992. **11**(3): p. 237-248.
567. Shoji, M., et al., *Molecular mechanisms linking thrombosis and angiogenesis in cancer.* Trends in cardiovascular medicine, 1997. **7**(2): p. 52-59.
568. Cao, R., et al., *PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis.* Cancer cell, 2004. **6**(4): p. 333-345.
569. Ahamed, J., M. Belting, and W. Ruf, *Regulation of tissue factor–induced signaling by endogenous and recombinant tissue factor pathway inhibitor 1.* Blood, 2005. **105**(6): p. 2384-2391.
570. Bootle-Wilbraham, C.A., et al., *Fibrinogen E-fragment inhibits the migration and tubule formation of human dermal microvascular endothelial cells in vitro.* Cancer research, 2000. **60**(17): p. 4719-4724.
571. Dirix, L., et al., *Plasma fibrin D-dimer levels correlate with tumour volume, progression rate and survival in patients with metastatic breast cancer.* British journal of cancer, 2002. **86**(3): p. 389.
572. Clark, R., et al., *Transient functional expression of alphaVbeta 3 on vascular cells during wound repair.* The American journal of pathology, 1996. **148**(5): p. 1407.
573. Maekawa, H., H. Sato, and D.M. Tollefsen, *Thrombin inhibition by HCII in the presence of elastase-cleaved HCII and thrombin–HCII complex.* Thrombosis research, 2000. **100**(5): p. 443-451.
574. Trousseau, A., *Phlegmasia Alba Dolens: Clinique Medicale de l-Hotel-Dieude Paris*. 1865, New Sydenham Society, London, UK.
575. SIMPSON-HAIDARIS, P. and B. Rybarczyk, *Tumors and fibrinogen.* Annals of the New York Academy of Sciences, 2001. **936**(1): p. 406-425.

576. Dvorak, H.F., D.R. Senger, and A.M. Dvorak, *Fibrin as a component of the tumor stroma: origins and biological significance.* Cancer and Metastasis Reviews, 1983. **2**(1): p. 41-73.
577. Dvorak, H.F., et al., *Human Breast Carcinoma: Fibrin Deposits ans Desmoplasia. Inflammatory Cell Type and Distribution. Microvasculature and Infarction 2 3.* Journal of the National Cancer Institute, 1981. **67**(2): p. 335-345.
578. Freedman, R.S., et al., *Peritoneal inflammation–A microenvironment for Epithelial Ovarian Cancer (EOC).* Journal of Translational Medicine, 2004. **2**: p. 23.
579. Sandoval, P., et al., *Carcinoma-associated fibroblasts derive from mesothelial cells via mesothelial-to-mesenchymal transition in peritoneal metastasis.* The Journal of pathology, 2013. **231**(4): p. 517-531.
580. Ronnov-Jessen, L., O.W. Petersen, and M.J. Bissell, *Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction.* Physiological reviews, 1996. **76**(1): p. 69-125.
581. Desmoulière, A. *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing*. in *2nd Scar meeting*. 2008.
582. Nagy, J.A., et al., *Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1989. **948**(3): p. 305-326.
583. Saposnik, B., et al., *A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis.* Blood, 2004. **103**(4): p. 1311-1318.
584. Qu, D., et al., *The Ser219–> Gly dimorphism of the endothelial protein C receptor contributes to the higher soluble protein levels observed in individuals with the A3 haplotype.* Journal of Thrombosis and Haemostasis, 2006. **4**(1): p. 229-235.
585. Xu, J., et al., *Metalloproteolytic release of endothelial cell protein C receptor.* Journal of Biological Chemistry, 2000. **275**(8): p. 6038-6044.
586. Fukudome, K., et al., *The endothelial cell protein C receptor cell surface expression and direct ligand binding by the soluble receptor.* Journal of Biological Chemistry, 1996. **271**(29): p. 17491-17498.
587. Zheng, X., et al., *Effects of membrane and soluble EPCR on the hemostatic balance and endotoxemia in mice.* Blood, 2007. **109**(3): p. 1003-1009.
588. Saposnik, B., et al., *Alternative mRNA is favored by the A3 haplotype of the EPCR gene PROCR and generates a novel soluble form of EPCR in plasma.* Blood, 2008. **111**(7): p. 3442-3451.
589. Beaulieu, L.M. and F.C. Church, *Activated protein C promotes breast cancer cell migration through interactions with EPCR and PAR-1.* Experimental cell research, 2007. **313**(4): p. 677-687.
590. Azzazene, D., et al., *Plasma endothelial protein C receptor influences innate immune response in ovarian cancer by decreasing the population of natural killer and TH17 helper cells.* International journal of oncology, 2013. **43**(4): p. 1011-1018.
591. Ducros, E., et al., *Soluble endothelial protein C receptor (sEPCR) is likely a biomarker of cancer-associated hypercoagulability in human hematologic malignancies.* Cancer medicine, 2012. **1**(2): p. 261-267.

592. Tsuneyoshi, N., et al., *Expression and anticoagulant function of the endothelial cell protein C receptor (EPCR) in cancer cell lines.* Thrombosis and haemostasis, 2001. **85**(2): p. 356-361.
593. Wang, X., et al., *Ovarian cancer, the coagulation pathway, and inflammation.* Journal of translational medicine, 2005. **3**(1): p. 1.
594. Scheffer, G.L., et al., *Expression of the vascular endothelial cell protein C receptor in epithelial tumour cells.* European journal of cancer, 2002. **38**(11): p. 1535-1542.
595. Ducros, E., et al., *Endothelial protein C receptor expressed by ovarian cancer cells as a possible biomarker of cancer onset.* International journal of oncology, 2012. **41**(2): p. 433-440.
596. Dahlbäck, B. and B. Villoutreix, *Molecular recognition in the protein C anticoagulant pathway.* Journal of thrombosis and haemostasis, 2003. **1**(7): p. 1525-1534.
597. Li, W., et al., *Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin.* Journal of Thrombosis and Haemostasis, 2005. **3**(7): p. 1351-1359.
598. Taylor, F.B., et al., *Endothelial cell protein C receptor plays an important role in protein C activation in vivo.* Blood, 2001. **97**(6): p. 1685-1688.
599. Kelemen, E., I. Cserhati, and B. Tanos, *Demonstration and some properties of human thrombopoietin in thrombocythaemic sera.* Acta haematologica, 1958. **20**(6): p. 350-355.
600. Broudy, V.C., N. Lin, and K. Kaushansky, *Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro.* Blood, 1995. **85**(7): p. 1719-1726.
601. Deutsch, V.R. and A. Tomer, *Megakaryocyte development and platelet production.* British journal of haematology, 2006. **134**(5): p. 453-466.
602. Kaushansky, K., et al., *Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development.* Proceedings of the National Academy of Sciences, 1995. **92**(8): p. 3234-3238.
603. Sungaran, R., B. Markovic, and B. Chong, *Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization.* Blood, 1997. **89**(1): p. 101-107.
604. Sohma, Y., et al., *Molecular cloning and chromosomal localization of the human thrombopoietin gene.* FEBS letters, 1994. **353**(1): p. 57-61.
605. Foster, D.C., et al., *Human thrombopoietin: gene structure, cDNA sequence, expression, and chromosomal localization.* Proceedings of the National Academy of Sciences, 1994. **91**(26): p. 13023-13027.
606. Gurney, A.L., et al., *Genomic structure, chromosomal localization, and conserved alternative splice forms of thrombopoietin.* Blood, 1995. **85**(4): p. 981-988.
607. Marcucci, R. and M. Romano, *Thrombopoietin and its splicing variants: structure and functions in thrombopoiesis and beyond.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2008. **1782**(7): p. 427-432.
608. Sirotkin, A., et al., *Thrombopoietin regulates proliferation, apoptosis, secretory activity and intracellular messengers in porcine ovarian follicular*

cells: involvement of protein kinase A. Journal of endocrinology, 2004. **183**(3): p. 595-604.
609. Sasaki, Y., et al., *Production of thrombopoietin by human carcinomas and its novel isoforms.* Blood, 1999. **94**(6): p. 1952-1960.
610. Furuhashi, M., Y. Miyabe, and H. Oda, *A case of thrombopoietin-producing ovarian carcinoma confirmed by immunohistochemistry.* Gynecologic oncology, 1999. **74**(2): p. 278-281.
611. Tsukishiro, S., et al., *Preoperative serum thrombopoietin levels are higher in patients with ovarian cancer than with benign cysts.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2008. **140**(1): p. 67-70.
612. Kaser, A., et al., *Interleukin-6 stimulates thrombopoiesis through thrombopoietin: role in inflammatory thrombocytosis.* Blood, 2001. **98**(9): p. 2720-2725.
613. Columbyova, L., M. Loda, and D.T. Scadden, *Thrombopoietin receptor expression in human cancer cell lines and primary tissues.* Cancer research, 1995. **55**(16): p. 3509-3512.
614. Otsuji, E., et al., *Outcome of surgical treatment for patients with scirrhous carcinoma of the stomach.* The American journal of surgery, 2004. **188**(3): p. 327-332.
615. Yonemura, Y., et al., *Surgical treatment for peritoneal carcinomatosis from gastric cancer.* European Journal of Surgical Oncology (EJSO), 2010. **36**(12): p. 1131-1138.
616. Huh, H.J., et al., *Prognostic significance of multidrug resistance gene 1 (MDR1), multidrug resistance-related protein (MRP) and lung resistance protein (LRP) mRNA expression in acute leukemia.* Journal of Korean medical science, 2006. **21**(2): p. 253-258.
617. Vtorushin, S., et al., *The phenomenon of multi-drug resistance in the treatment of malignant tumors.* Exp Oncol, 2014. **36**(3): p. 144-56.
618. Sui, H., et al., *Epithelial-mesenchymal transition and drug resistance: role, molecular mechanisms, and therapeutic strategies.* Oncology research and treatment, 2014. **37**(10): p. 584-589.
619. Nieto, M.A., *The ins and outs of the epithelial to mesenchymal transition in health and disease.* Annual review of cell and developmental biology, 2011. **27**: p. 347-376.
620. Lee, J.M., et al., *The epithelial–mesenchymal transition: new insights in signaling, development, and disease.* The Journal of cell biology, 2006. **172**(7): p. 973-981.
621. Kalluri, R. and E.G. Neilson, *Epithelial-mesenchymal transition and its implications for fibrosis.* Journal of Clinical Investigation, 2003. **112**(12): p. 1776.
622. Yashiro, M. and T. Matsuoka, *Scirrhous gastric cancer: a critical review.* OA Cancer, 2014. **2**(4).
623. Buø, L., et al., *The fibrinolytic system in human ascites.* Scandinavian journal of gastroenterology, 1995. **30**(11): p. 1101-1107.
624. Secchi, M.F., et al., *Recent data concerning heparanase: focus on fibrosis, inflammation and cancer.* Biomolecular concepts, 2015. **6**(5-6): p. 415-421.

625. Wood, R.J. and M.D. Hulett, *Cell surface-expressed cation-independent mannose 6-phosphate receptor (CD222) binds enzymatically active heparanase independently of mannose 6-phosphate to promote extracellular matrix degradation.* Journal of Biological Chemistry, 2008. **283**(7): p. 4165-4176.
626. Nakajima, M., T. Irimura, and G.L. Nicolson, *Heparanases and tumor metastasis.* Journal of cellular biochemistry, 1988. **36**(2): p. 157-167.
627. Cohen, T., et al., *VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells.* Journal of Biological Chemistry, 1995. **270**(19): p. 11322-11326.
628. Vlodavsky, I., et al., *Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism?* Trends in biochemical sciences, 1991. **16**: p. 268-271.
629. Goldshmidt, O., et al., *Heparanase mediates cell adhesion independent of its enzymatic activity.* The FASEB Journal, 2003. **17**(9): p. 1015-1025.
630. Fux, L., et al., *Heparanase: busy at the cell surface.* Trends in biochemical sciences, 2009. **34**(10): p. 511-519.
631. Koliopanos, A., et al., *Heparanase expression in primary and metastatic pancreatic cancer.* Cancer research, 2001. **61**(12): p. 4655-4659.
632. HIROKIKUNIYASU, Y.C., T. KUBOZOE, and T. TAKAHASHI, *Co-expression of CD44v3 and heparanase is correlated with metastasis of human colon cancer.* International journal of molecular medicine, 2002. **10**: p. 333-337.
633. Uno, F., et al., *Antisense-mediated suppression of human heparanase gene expression inhibits pleural dissemination of human cancer cells.* Cancer research, 2001. **61**(21): p. 7855-7860.
634. Marchetti, D., et al., *FGF2 binding, signaling and angiogenesis are modulated by heparanase in metastatic melanoma cells.* Melanoma Research, 2006. **16**: p. S67.
635. Zhang, J., et al., *A cationic-independent mannose 6-phosphate receptor inhibitor (PXS64) ameliorates kidney fibrosis by inhibiting activation of transforming growth factor-β1.* PloS one, 2015. **10**(2): p. e0116888.
636. Leksa, V., et al., *Dissecting mannose 6-phosphate-insulin-like growth factor 2 receptor complexes that control activation and uptake of plasminogen in cells.* Journal of Biological Chemistry, 2012. **287**(27): p. 22450-22462.
637. Dennis, P.A. and D.B. Rifkin, *Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor.* Proceedings of the National Academy of Sciences, 1991. **88**(2): p. 580-584.
638. Ishai-Michaeli, R., A. Eldor, and I. Vlodavsky, *Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix.* Cell regulation, 1990. **1**(11): p. 833-842.
639. Lai, W.T., V. Krishnappa, and D.G. Phinney, *Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels.* Stem cells, 2011. **29**(7): p. 1102-1111.

640. Wu, J., et al., *Characterization of site-specific glycosylation of secreted proteins associated with multi-drug resistance of gastric cancer.* Oncotarget, 2016.
641. She, J.-J., et al., *Side population cells isolated from KATO Ⅲ human gastric cancer cell line have cancer stem cell-like characteristics.* World J Gastroenterol, 2012. **18**(33): p. 4610-4617.
642. Crocker, P.R., *Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell–cell interactions and signalling.* Current opinion in structural biology, 2002. **12**(5): p. 609-615.
643. Fu-Tong, L. and G.A. Rabinovich, *Galectins as modulators of tumour progression.* Nature reviews. Cancer, 2005. **5**(1): p. 29.
644. Wang, C., et al., *Transforming growth factor alpha (TGFα) regulates granulosa cell tumor (GCT) cell proliferation and migration through activation of multiple pathways.* PLoS One, 2012. **7**(11): p. e48299.
645. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.* Genes & development, 2003. **17**(10): p. 1253-1270.
646. Toma, J.G., et al., *Isolation of multipotent adult stem cells from the dermis of mammalian skin.* Nature cell biology, 2001. **3**(9): p. 778-784.
647. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer research, 2003. **63**(18): p. 5821-5828.
648. Song, S., et al., *Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs.* Proceedings of the National Academy of Sciences, 2000. **97**(15): p. 8658-8663.
649. Blum, J.L., et al., *Phase II trial of capecitabine and weekly paclitaxel as first-line therapy for metastatic breast cancer.* Journal of clinical oncology, 2006. **24**(27): p. 4384-4390.
650. McCubrey, J.A., et al., *GSK-3 as potential target for therapeutic intervention in cancer.* Oncotarget, 2014. **5**(10): p. 2881.
651. Takagaki, K., et al., *Role of Chk1 and Chk2 in Ara-C-induced differentiation of human leukemia K562 cells.* Genes to Cells, 2005. **10**(2): p. 97-106.
652. Cantó, C., et al., *AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity.* Nature, 2009. **458**(7241): p. 1056.
653. Wisdom, R., R.S. Johnson, and C. Moore, *c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms.* The EMBO journal, 1999. **18**(1): p. 188-197.
654. Bossy-Wetzel, E., L. Bakiri, and M. Yaniv, *Induction of apoptosis by the transcription factor c-Jun.* The EMBO journal, 1997. **16**(7): p. 1695-1709.
655. Li, H., et al., *Suramin inhibits cell proliferation in ovarian and cervical cancer by downregulating heparanase expression.* Cancer cell international, 2015. **15**(1): p. 52.
656. Weidle, U.H., et al., *Mechanisms and Targets Involved in Dissemination of Ovarian Cancer.* Cancer Genomics-Proteomics, 2016. **13**(6): p. 407-423.
657. Kenny, H.A., et al., *Mesothelial cells promote early ovarian cancer metastasis through fibronectin secretion.* The Journal of clinical investigation, 2014. **124**(10): p. 4614-4628.
658. Cannistra, S.A., *Cancer of the ovary.* New England Journal of Medicine, 1993. **329**(21): p. 1550-1559.

659. Davidowitz, R., et al., *Identification of mechanism involved in mesothelial clearance by ovarian tumor spheroids*. 2011, AACR.
660. Slack-Davis, J., et al., *VCAM-1 regulates ovarian cancer mesothelial invasion and peritoneal metastasis*. 2008, AACR.
661. Takatsuki, H., et al., *Adhesion of gastric carcinoma cells to peritoneum mediated by α3β1 integrin (VLA-3).* Cancer research, 2004. **64**(17): p. 6065-6070.
662. Bourboulia, D. and W.G. Stetler-Stevenson. *Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion*. in *Seminars in cancer biology*. 2010. Elsevier.
663. Heino, J., et al., *Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit.* Journal of Biological Chemistry, 1989. **264**(1): p. 380-388.
664. Honjo, K., et al., *Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice.* The FASEB Journal, 2017. **31**(6): p. 2625-2637.
665. Matte, I., et al., *Ovarian cancer ascites enhance the migration of patient-derived peritoneal mesothelial cells via cMet pathway through HGF-dependent and-independent mechanisms.* International journal of cancer, 2015. **137**(2): p. 289-298.
666. Bekes, I., et al., *Does VEGF facilitate local tumor growth and spread into the abdominal cavity by suppressing endothelial cell adhesion, thus increasing vascular peritoneal permeability followed by ascites production in ovarian cancer?* Molecular cancer, 2016. **15**(1): p. 13.
667. Mutsaers, S.E., *Mesothelial cells: their structure, function and role in serosal repair.* Respirology, 2002. **7**(3): p. 171-191.
668. Hassan, R. and M. Ho, *Mesothelin targeted cancer immunotherapy.* European journal of cancer, 2008. **44**(1): p. 46-53.
669. Chu, A.Y., et al., *Utility of D2-40, a novel mesothelial marker, in the diagnosis of malignant mesothelioma.* Modern Pathology, 2005. **18**(1): p. 105.
670. Amin, K.M., et al., *Wilms' tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma.* The American journal of pathology, 1995. **146**(2): p. 344.
671. Ip, C.K., et al., *Hepatocyte growth factor modulates adhesion of ovarian cancer cells to the peritoneum mesothelium through p70 S6 kinase*. 2011, AACR.
672. Kuai, W.-X., et al., *Interleukin-8 associates with adhesion, migration, invasion and chemosensitivity of human gastric cancer cells.* World journal of gastroenterology: WJG, 2012. **18**(9): p. 979.
673. Chin, B.S., et al., *Interleukin-6, tissue factor and von Willebrand factor in acute decompensated heart failure: relationship to treatment and prognosis.* Blood coagulation & fibrinolysis, 2003. **14**(6): p. 515-521.
674. Kerr, R., D. Stirling, and C.A. Ludlam, *Interleukin 6 and haemostasis.* British journal of haematology, 2001. **115**(1): p. 3-12.