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MESENCHYMAL STEM CELLS IN VASCULAR STRUCTURE AND REMODELING IN CANCER

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Mesenchymal stem cells in vascular structure and remodeling in cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Mesenchymal stem cells (MSCs) are present in vascular structure and play an important role in vessel remodeling in normal, as well as pathological conditions, such as transplant arteriosclerosis or tumor angiogenesis. Moreover, MSCs affect tumor growth and metastasis by production of chemokines, such as (C-C motif) ligand 7 (CCL7). Similar effect on tumor biology exert multiple other factors, such as CCAAT-enhancer binding protein β (C/EBPβ), which is a transcription factor playing an essential role in mammary gland development and breast cancer progression.

In **study I**, presence of MSCs in vascular structure, as well as their role in vascular remodeling in transplant arteriosclerosis was analyzed. Specifically, rat allograft model was used to identify the predominant cell types associated with this process and to find factors crucial for their recruitment into the graft. This study identified adventitia as a potentially important source of mesenchymal stem cells that contribute to formation of intimal hyperplasia. Furthermore, monocyte chemoattractant protein-1 (MCP-1) was found as a potent chemokine for the recruitment of adventitial vascular progenitor cells to intimal lesions.

Study II and III focus on the role of C/EBPβ transcription factor in breast cancer progression. Namely, in study II C/EBPβ was associated with epithelial-to-mesenchymal transition (EMT) features in triple-negative human breast cancer and invasive areas of mammary tumors in MMTV-PyMT mice. *In vitro* studies showed that C/EBPβ was repressed during EMT by miR-155, a breast cancer oncomiR. Moreover, C/EBPβ depletion enhanced TGFβ response towards EMT and contributed to evasion of growth inhibitory response to TGFβ. C/EBPβ loss caused potentiated invasion and metastasis of breast cancer cells to the lungs in mouse 4T1 model. In addition, C/EBPβ was shown to transcriptionally activate genes encoding epithelial junction proteins E-cadherin and coxsackie virus and adenovirus receptor (CAR). Study III was focused on explaining the mechanism of C/EBPβ effect on metastasis, as well as determining the relationship between C/EBPβ expression and survival of breast cancer patients. Firstly, study showed that decrease in C/EBPβ expression was associated with shorter overall survival of breast cancer patients. Next, loss of C/EBPβ affected tumor growth, morphology and lung metastasis in murine 4T1 breast cancer model. Moreover, inhibition of C/EBPβ caused enhanced histocompatibility complex II (MHCII) expression and accumulation of CD45+, CD3+ and CD4+ lymphocytes in the tumors. Additional experiments confirmed the role of inflammation in C/EBPβ-mediated metastasis formation.

Study IV involved analysis of crosstalk between MSCs and colon cancer. Results demonstrated that MSCs affect CT26 tumor cell proliferation, migration and expression of different chemokines in coculture with CT26 cells *in vitro*, such as (C-C motif) ligand 7 (CCL7). The next goal of the study was to analyze the effect of CCL7 overexpression on tumor progression in mouse CT26 colon cancer model. Cells overexpressing CCL7

accelerated early phase of tumor growth and led to higher lung metastasis rate in tumor-bearing mice. Lastly, higher CCR2 expression, which is a CCL7 receptor, was associated with shorter overall survival of colorectal cancer patients.

LIST OF SCIENTIFIC PAPERS

Included in the thesis:

I. Monika K. Grudzinska*, **Ewa Kurzejamska***, Krzysztof Bojakowski, Joanna Soin, Michael H. Lehmann, Hans Reinecke, Charles E. Murry, Cecilia Söderberg-Nauclér and Piotr Religa. Monocyte Chemoattractant Protein 1–Mediated Migration of Mesenchymal Stem Cells Is a Source of Intimal Hyperplasia. Arterioscler Thromb Vasc Biol. 2013 Jun;33(6):1271-9. doi: 10.1161/ATVBAHA.112.300773.

II. Joel Johanssson*, Tove Berg*, **Ewa Kurzejamska**, Mei-Fong Pang, Vedrana Tabor, Malin Jansson, Pernilla Roswall, Kristian Pietras, Malin Sund, Piotr Religa and Jonas Fuxe. MiR-155-mediated loss of C/EBPβ shifts the TGF-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer. Oncogene. 2013 Dec 12;32(50):5614-24. doi: 10.1038/onc.2013.322. Epub 2013 Aug 19.

III. **Ewa Kurzejamska**, Joel Johansson, Karin Jirström, Varsha Prakash, Sharan Ananthaseshan, Louis Boon, Jonas Fuxe and Piotr Religa. C/EBPβ expression is an independent predictor of overall survival in breast cancer patients by MHCII/CD4-dependent mechanism of metastasis formation. Oncogenesis. 2014 Nov 3;3:e125. doi: 10.1038/oncsis.2014.38.

IV. **Ewa Kurzejamska**, Natalia Landázuri, Mariusz Sacharczuk, Oksana Kovtonyuk, Marzena Łazarczyk, Sharan Ananthaseshan, Hanna Menkens and Piotr Religa. Chemokine (C-C motif) ligand 7 (CCL7) affects lung metastasis in colon cancer. *Manuscript*.

Others not included in the thesis:

I. Mensur Dzabic, Krzysztof Bojakowski, **Ewa Kurzejamska**, Grzegorz Styczyński, Piotr Andziak, Cecilia Söderberg-Nauclér and Piotr Religa. Significance of cytomegalovirus infection in the failure of native arteriovenous fistula. Clin Microbiol Infect. 2012 Jan;18(1):E5-7. doi: 10.1111/j.1469-0691.2011.03691.x.

II. Krzysztof Bojakowski, Mensur Dzabic, **Ewa Kurzejamska**, Grzegorz Styczyński, Piotr Andziak, Zbigniew Gaciong, Cecilia Söderberg-Nauclér C and Piotr Religa. A high red blood cell distribution width predicts failure of arteriovenous fistula. PLoS One. 2012;7(5):e36482. doi: 10.1371/journal.pone.0036482.

III. Alice Assinger, Julia B. Kral, Koon-Chu Yaiw, Waltraud C. Schrottmaier, **Ewa Kurzejamska,** Yajuan Wang, Abdul-Aleem Mohammad, Piotr Religa, Afsar Rahbar, Gernot Schabbauer, Lynn M. Butler and Cecilia Söderberg-Nauclér. Arterioscler Thromb Vasc Biol. 2014 Apr;34(4):801-9. doi: 10.1161/ATVBAHA.114.303287.

** equal contribution*

CONTENTS

LIST OF ABBREVIATIONS

CAR	coxsackie virus and adenovirus receptor
CCL7	chemokine (C-C motif) ligand 7
CCR1	chemokine (C-C) motif receptor 1
CCR2	chemokine (C-C) motif receptor 2
CCR3	chemokine (C-C) motif receptor 3
CD	cluster of differentiation
C/EBPβ	CCAAT-enhancer binding protein beta
ECM	extracellular matrix
EM	electron microscopy
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
HER2	human epidermal growth factor receptor 2
IPγ-10	interferon γ-induced protein 10
MCP-1	monocyte chemoattractant protein 1
MHCII	major histocompatibility complex II
MSC	mesenchymal stem cell
NMuMG	namru mouse mammary gland
PyMT	polyoma virus middle-T
PCR	polymerase chain reaction
PR	progesterone receptor
RANTES	regulated upon activation, normally T-expressed, and presumably secreted
RT PCR	real time PCR
SDF-1	stromal-derived factor 1
SMC	smooth muscle cell
TGFβ	transforming growth factor beta
TMA	tissue microarray

1 INTRODUCTION

1.1 BLOOD VESSELS

Blood vessels provide circulatory system for the nutrients and oxygen in the body, ensuring normal tissue function. All vessels except for the capillaries contain three layers: tunica intima, tunica media and tunica adventitia (Fig. 1). Tunica intima, the thinnest layer in the vessel, is composed of a single sheet of squamous endothelial cells covering the lumen, which are surrounded by elastic connective tissue called internal elastic lamina. Endothelial cells rest on a basement membrane composed of extracellular matrix proteins, such as collagen type IV, heparin sulfate proteoglycans and laminin [1]. Tunica media is rich in vascular SMCs and represents the thickest layer in the arteries. SMCs not only produce multiple ECM components (collagen, elastic etc.), but also regulate the caliber of the blood vessels in the body and thus, affect local blood pressure. Tunica adventitia is usually thickest layer in the veins and entirely comprises of loose connective tissue containing mainly collagenous and elastic fibres, fibroblasts and macrophages. Additionally, it involves nerves supplying the vessels and nutrient capillaries (vasa vasorum) in case of larger blood vessels. Adventitia has been shown to play a crucial role in vascular remodeling and development of vascular diseases, including atherosclerosis, transplant vasculopathy, hypertension as well as restenosis [2-4].

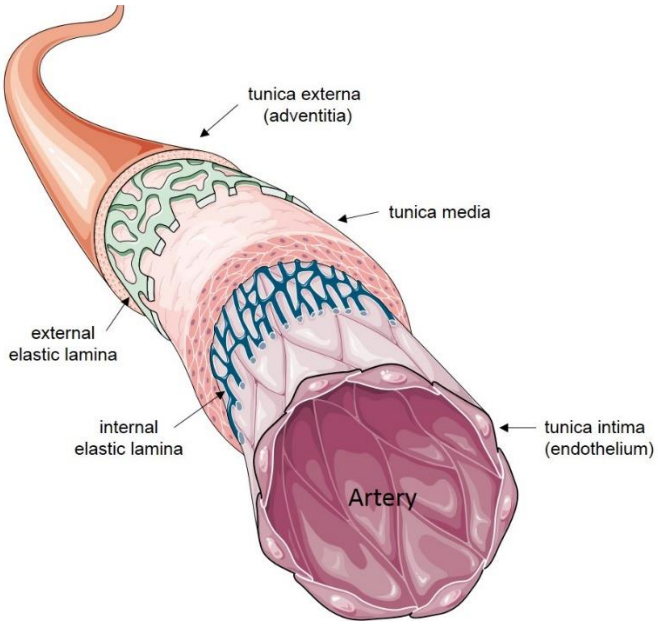

Fig. 1 Structure of the arterial wall.

1.1.1 Vascular remodeling

Vascular remodeling is a complex process of structural changes caused by alterations in various cellular processes, such as cell growth, migration or ECM modifications and depends on the interplay between produced growth factors, vasoactive substances and hemodynamic stimuli [5]. Even though remodeling is usually an adaptive response to long-term changes in hemodynamic conditions [6], it can also provide background for cardiovascular diseases, such as transplant arteriosclerosis, restenosis, atherosclerosis, hypertension or diabetes.

Vascular architecture can be altered in multiple manners dependent on different stimuli. For example, in response to increased pressure in the artery, the wall/lumen width ratio changes, at the same time enhancing vessel reactivity, what results in augmented peripheral resistance typical for hypertension [7]. Reduced lumen diameter is also related to vascular remodeling in other diseases, such as restenosis after percutaneous intervention or cardiac allograft vasculopathy [6]. Conversely, remodeling can also lead to higher lumen diameter and can compensate for increased plaque load [8].

Vessel remodeling can also involve changes in the dimensions of the lumen, caused by active reorganization of wall components. Such alterations are observed in cases of vascular dilatation related to sustained high blood flow, what often occurs in patients with arteriovenous fistula and was shown in animal models [9, 10], as well as in case of cell apoptosis and matrix proteolysis leading to formation of aneurysm [11, 12].

Moreover, blood vessels undergo remodeling as a result of different forms of vascular injury, including transplantation. Mechanical injury can result with vessel constriction and smaller vascular lumen in response to the scarring in the outer vessel layer. Main features of transplant vasculopathy include inflammation and formation of intimal hyperplasia.

1.1.1.1 Intimal hyperplasia

Intimal hyperplasia is associated with increased cell number and amount of ECM in the intimal layer of the vessel. Physiologically, it occurs during closure of ductus arteriosus [13], involution of the uterus [14] and aging of human aorta [15]. Pathologically, intimal hyperplasia occurs in pulmonary hypertension [16], after balloon angioplasty [17], transplantation [18], in veins used as arteriovenous fistulas or artery bypass conduits [19] and finally, in pre-atherosclerotic lesions [20].

Development of intimal hyperplasia requires a few essential stages. One of them is recruitment of inflammatory cells, such as macrophages and leukocytes, to an injured artery and mobilization of vascular progenitor cells from their niches. Platelets, activated endothelial cells, smooth muscle cells and macrophage foam cells produce platelet-derived growth factor (PDGF) and additional factors [1], which act as a chemoattractants enabling migration of phenotypically modified SMCs from the media into the neointima and their proliferation [21]. Moreover, PDGF stimulates production of proteoglycans and collagen. At the same time, metalloproteinases (MMPs) cause remodeling of ECM, what further facilitates SMC migration and migration. Next, intima grows in response to cell proliferation, increased synthesis of ECM [21], progressive apoptosis and fibrosis in media.

Cells forming neointima have been reported to originate from different sources. Namely, they can be derived from the adventitia, for example fibroblasts, pericytes, vascular progenitor cells [2, 22] or be circulating progenitor cells, such as bone marrow–derived cells, endothelial progenitor cells or smooth muscle progenitor cells [23-25].

1.1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells having ability to self-renewal and differentiation into several cell types, such as osteoblasts, chondrocytes, myocytes and adipocytes [26]. MSCs were first isolated and amplified from bone marrow in 1970 [27]. In order to standardize

isolation of human MSCs, The International Society for Cell therapy established a set of criteria, including adherence to plastic in *in vitro* culture, expression of a variety of surface markers, such as CD73, CD90, and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79, or CD19 surface molecules as assessed by fluorescence-activated cell sorting analysis, and capacity to differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [28].

MSCs can be isolated from a variety of tissues, such as bone marrow [26, 29], adipose tissue [30], lung tissue [31], synovial tissue [32], umbilical cord blood [33] and peripheral blood [34]. Even though they display different growth rates, they share capability to mesodermal differentiation and similar surface markers [35, 36]. Moreover, the fact that MSCs have been isolated from several tissue types of adult mice, implicates the possibility that MSCs reside in all postnatal organs [37].

MSCs were shown not only to differentiate into mesoderm-derived tissue, but also ectoderm-derived tissue like astrocytes [38] or endoderm-derived cells and cardiomyocytes [39, 40]. Apart from their differentiation properties, MSCs suppress proliferation of T-lymphocytes [41] and have ability to engraft, differentiate and contribute to functional recovery of ischaemic [42, 43], as well as infarcted [44] swine myocardium. Hence, MSCs have been used as a therapy in clinical trials for ischemic heart disease [45-47].

Over the last decade, vascular biology field focused on analyzing the role of different progenitor cells, including mesenchymal stem cells in the formation of blood vessels [48]. Blood vessels are a source of stem and progenitor cells, what affect various vascular processes and diseases [49]. Furthermore, vessels are closely associated with stem cell niches regulating and protecting different stem and progenitor cells. Multiple studies have shown that mural cells, like smooth muscle cells or pericytes, are recruited to vessels from local areas [50-53]. Since MSCs are important part of perivascular niche in several human organs [54] and coexpress similar markers as pericytes, discussion arose about the relationship between those cell types. Pericytes not only contribute to vessel stabilization by interactions with endothelial cells, but are a source of mesenchymal stem cells and differentiate into cells originating from mesenchyme [55].

Increasing body of evidence supports association of vascular progenitor cells with intimal hyperplasia – a process of universal response of a vessel to injury [48, 56]. Namely, Sca-1+ cells of adventitial origin have been shown to increase their number and proliferation rate during neointima formation in the injury model [57]. Furthermore, MSCs and pericyte-like cells contribute to restenosis after arterial injury [58]. In addition, adventitial progenitor cells are able to differentiate into SMCs that subsequently contribute to atherosclerosis of vein grafts in ApoE-deficient mice [2].

1.2 CANCER

Cancer, also called malignant neoplasm, is one of the major health problems in the recent world. This term includes a broad group of diseases that cause loss of health and severe disability of the patients. It is characterized by uncontrollable cell growth and divisions, what leads to formation of malignant tumors and invasion to the other parts of the body. Invasion and metastasis do not happen in all tumor cases though, as some tumors, referred to as benign, do not cause distant metastasis. Invasive tumor cells might spread through the bloodstream or lymphatic system and form metastasis in different organs.

In contrast to other cells, the growth of neoplastic cells is autonomous, excessive and disorganized. Typical hallmarks of cancer include: self-sufficiency in growth signals, apoptosis evasion, insensitivity to anti-growth signals, sustained angiogenesis, limitless potential and tissue invasion and metastasis (Fig. 2) [59].

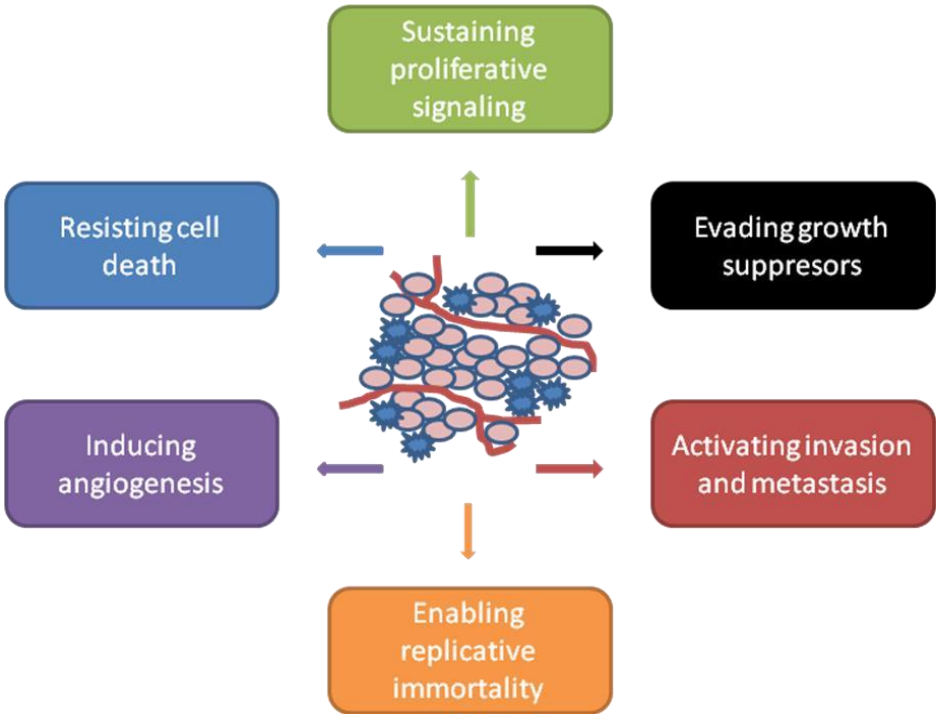

Fig. 2 Hallmarks of cancer according to Hanahan & Weinberg (modified) [59].

There are several different types of malignancies that affect humans and they are classified based on the type of cell that the tumor cells resemble, which is hence assumed to be the tumor origin. These types include:

- carcinomas: group of cancers originating from epithelial cells, including the most common cancers such as breast, colon, lung or prostate cancer,

- sarcomas: neoplasm derived from connective tissue (e.g. bone, cartilage, fat), originating from mesenchymal cells outside the bone marrow,

- lymphomas and leukemias: neoplasm arising from blood cells that leave the bone marrow and mature in lymph nodes and blood.

1.2.1 Breast cancer progression

Breast cancer is the most common cancer among women and accounts for 25% of its all cases [60]. In 2012, it resulted in 1.68 million cases and 522000 deaths [60]. Although male breast cancer is more than 100 times less frequent, men have usually lower survival rates than women as a result of delayed diagnosis and more advanced disease at presentation [61].

It is very hard to determine direct causes of breast cancer, as it is a very complex disease. There

are many factors which are associated with breast cancer risk, e.g. lack of childbearing or breastfeeding [62], higher endogenous estrogen levels [63], high fat diet, older age, smoking, viral infections, radiation, obesity and other environmental agents.

Breast cancer classification is based on several grading systems, which contribute to patient's prognosis as well as prediction of treatment response. Classification of the tumors can be assessed by means of its histological appearance. Breast cancer arises from epithelial parts of breast tissue, usually either from the inner lining of milk ducts or the lobules supplying ducts with milk and is referred to as ductal or lobular carcinoma, respectively. Additionally, breast carcinomas may be divided into carcinomas *in situ*, which are low-grade tumors that do not spread to the surrounding tissue, or invasive carcinomas, where cancer cells break through the wall of the milk duct and invade breast tissue.

Progress in science during the recent years enabled categorizing tumors based on gene expression profiling with use of microarray analysis [64, 65]. Owing to mRNA profiling, tumors can be identified as different subtypes, including luminal A, luminal B, basal- and normal-like [64-66]. Majority of breast cancer subtypes are luminal, in which tumor cells resemble the inner epithelial cell lining of mammary ducts. Luminal A subtype is usually HER2- , ER+ and/or PR+ and due to its responsiveness to hormone therapy, has better prognosis, higher survival rates and lower recurrence when compared to other subtypes [67, 68]. However, some luminal A tumors carry p53 mutations, what contributes to their worse prognosis [68]. Luminal B tumors tend to be ER+ and/or PR+ as well, but they have large number of actively dividing tumor cells. Moreover, their prognosis is worse, compared with luminal A subtype, due to different factors, such as bigger tumor size, lymph node positivity and p53 mutations [67-70]. Basal-like tumors resemble myoepithelial cells lining basement membrane of the mammary duct [71] and usually are ER-, PR- and HER2-. They are more migratory, invasive and have a worse prognosis compared to luminal tumors [72-75]. Normal-like tumors constitute only 6-10% of all breast cancers, are often small and tend to have a good prognosis [75].

Some people have genetic mutations that make them more susceptible to breast cancer. However, only 5-10% of all breast cancer cases have a genetic background [76]. The most frequent gene defects are found in the *BRCA1* and *BRCA2* genes. Women who carry mutations in those genes have even up to an 80% chance of getting breast cancer. Other significant mutations include: *p53* (Li-Fraumeni syndrome), *PTEN* (Cowden syndrome), and *STK11* (Peutz–Jeghers syndrome), *CHEK2*, *ATM*, *BRIP1*, and *PALB2*.

Death of breast cancer patients is caused mainly by spreading cancer to distant organs. During cancer progression, cells escape anti-proliferative control by acquiring several mutations in tumor suppressor genes, such as *p53*, phosphatase and tensin homolog *Pten* or retinoblastoma protein (RB) [77], and secrete their own stimulatory factors, what helps them to divide in an uncontrolled manner. Attained mutations and balance between activity of oncogenes and tumor suppressors enables the cells to acquire invasive phenotype.

Breast cancer progression begins with attachment of tumor cells to the basement membrane, which is mediated by certain glycoproteins, like fibronectin and laminins [78]. Attached tumor cells, as well as other cells from tumor microenvironment release enzymes, such as matrix

metalloproteinases, which disrupt basement membrane and degrade the matrix [79]. Once tumor cells come in direct contact with remodeled stroma, their properties and behavior undergo modifications [80]. They start to recruit inflammatory cells, such as leukocytes, macrophages and neutrophils to the tumor stroma, which as a result produce cytokines [81]. Altogether, in order to invade, tumor cells lose cell-cell and cell-ECM interactions controlled by integrins and cadherins, and become more migratory owing to released chemokines and growth factors, and recruitment of stromal cells. Subsequently, invasive cancer cells migrate towards blood and lymphatic vessels and intravasate to the circulation [82]. Their capability to secrete pro-angiogenic factors leads to promoting angiogenesis (e.g. VEGF-A) and lymphangiogenesis (e.g. VEGF-C) [83]. As a consequence, new vessels produce chemokines and other stimulating factors for the tumor cells [84]. Since tumor blood vessels have abnormal structure and are usually not covered with pericytes and smooth muscle cells, what causes their leakage and high permeability, tumor cells are able to enter the circulation. Moreover, tumor cells can also intravasate to the lymphatic vessels, as they lack the tight inter-endothelial junctions typically seen in blood vessels, as well as the surrounding layers of pericytes, smooth muscle cells and basement membranes [85]. Finally, breast cancer cells which passed the intravasation barrier, are able to form metastases at distant sites, such as lung, bone and liver [86].

1.2.2 Colorectal cancer progression

Colorectal cancer is one of the leading cancer-related deaths worldwide Although survival rates of patients continue to increase with time, mainly as a result of improved diagnostics and treatment, 5-year survival remains below 60% in Europe [87]. Major cause of mortality in colorectal cancer patients is liver metastasis, either present already at cancer diagnosis stage, or developing after primary tumor resection.

Colonic epithelium consists of approximately ten million invaginations, called crypts. The base of the crypt contains dividing stem cells and multiplying daughter cells and is a starting point for the cell migration towards the epithelium surface, where the cells die and become replaced by continuously streaming new cells.

Histopathologic changes appear as a consequence of both genetic and environmental factors. Main environmental factors involve pathogen invasion, toxins, polyamines, ROS (reactive oxygen species) production and stress. Somatic and inherited mutations affect multiple genes, e.g. *APC* (adenomatous polyposis coli), *K-RAS*, β-catenin, *COX2* (cyclooxygenase-2), *SMAD4*, *TGF-βR2* (transforming growth factor-beta Receptor-Type II), *BAX* (Bcl2 associated-X protein), *MMP*s (matrix metalloproteinases), *E2F4* (E2F Transcription Factor-4), *MMR* (mismatch repair) genes and many others [88, 89]. Genomic instability enables cells to acquire necessary mutations to turn into a cancer cell [90].

Progression of majority of colorectal cancers involves multiple morphological stages. In the beginning, some of the crypts appear to accumulate cells at the surface, forming either hyperplastic or dysplastic tissue. As a result or excessive cell accumulation, polyps start to grow and protrude from epithelial surface. Dysplastic polyps, referred to as adenomas, become more dysplastic with time, while hyperplastic polyps become cancer mainly in a nonclassical manner.

The growth of adenomas is triggered by adverse conditions like bacterial or viral invasions, subsequently causing mutation in the APC (Adenomatous Polyposis Coli) regulatory pathway [91] usually affecting either APC or β-catenin. APC represses β-catenin, which reduces the tendency to abnormal tissue expansion by enhancing expression of proteins promoting cell division and affecting cell adhesion. As cells migrate from base crypts towards the epithelium surface, APC expression increases and inhibits β-catenin, what in turn promotes apoptosis at the surface and at the same time provides optimal balance in production from the crypt base.

COX2 mutations are responsible for transformation of dysplastic epithelium (stage I) to early adenoma phase (stage II) and appear in the majority of human colorectal adenocarcinomas. Next stage in progression involves mutations in *RAS* genes, where the most common gene affected is *K-RAS* and less commonly *H-RAS*. *K-RAS* mutations, next to common mutations in *DCC*, *MLH1* and *MSH2* enable the transition from early to late adenoma (stage III). Additionally, late adenomas undergo other type of mutations, like aneuploidy, loss of heterozygosity or altered DNA methylation, to name a few [90]. Finally, progression to tumor metastasis (stage IV), most commonly affecting liver, lungs, bone and brain, involves other genes, such as *BAX*, *E2F4*, *MSH3*, *MSH6*, *TGF-βR2*, *BAX* and *MMP*s, *p53* and *SMAD4*.

1.2.3 Metastasis

Metastasis is a complicated process, during which cancer cells spread from the primary tumor to the distant organs in the body (Fig. 3).

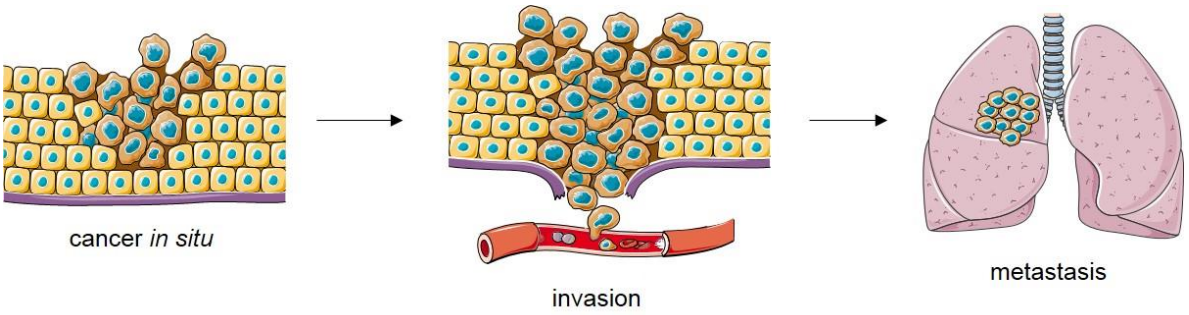

Fig. 3 Metastatic process.

In order to survive and proliferate in a new tissue microenvironment, cancer cells acquire certain properties and undergo a series of metastatic events [92]. This process is often referred to as metastatic cascade, which involves multiple steps and engagement of various signaling pathways [93, 94]. The most crucial events of the cascade are mobilization of cancer cells, followed by intravasation of the tumor vasculature and subsequently, extravasation and colonization at secondary sites.

1.2.3.1 Routes of metastatic spread

There are different routes, which tumor cells use in order to metastasize. The most typical one for sarcomas and some carcinomas (e.g. renal cell carcinoma) is hematogenous, in which tumor cells are transported within the blood circulation. For carcinomas, on the other hand, lymphatogenous spread is the most common and it allows tumor cells to reach lymph nodes and finally, other parts of the body. As lymphatics drain into systemic venous system, cancer cells can eventually enter

the bloodstream. Alternative route of metastasis occurs through seeding of body cavities, such as pleural, pericardial, peritoneal or subarachnoid cavities. For example, lung cancer can spread through the pleural cavity, while ovarian cancer might be transported transperitoneally to the liver surface.

1.2.3.2 Mechanisms of metastasis development

Currently, there are different theories explaining the mechanisms of development of distal hematogenous metastases. Namely, such metastases can result either from direct blood vessel intravasation at the primary site or originate from the tumor cells that have previously colonized local lymph nodes [92].

1.2.3.2.1 Intravasation

Intravasation initiates detachment of cancer cells from the primary tumor site and involves their invasion through the basal membrane into surrounding blood or lymphatic vessels [95]. Cancer cells can use both active and passive approaches to penetrate the vasculature.

Active intravasation assumes an active migration of tumor cells toward and into the closest blood vessel [96]. At the beginning, cancer cells adhere to venular endothelial cells, and then to the subendothelial basement membrane proteins, such as type IV and V collagen and laminin. Finally, tumor cells adhere to connective tissue components, for example type I collagen, fibronectin and hyaluronan, what contributes to tumor cell invasion into subendothelial stroma as well as growth at the site of metastasis [97]. Among the proteins contributing to intravasation is urokinase (uPA), an enzyme degrading extracellular matrix (ECM) elements and basement membrane. Moreover, uPA is responsible for activation of several growth factors and metalloproteinases (MMPs), which also degrade ECM, leading to tumor invasion and intravasation [98]. Additionally, studies have shown that tumor-associated macrophages, which are a part of metastatic tumor microenvironment [99, 100], increase tumor cell migration and intravasation by secretion of chemokinetic and chemotactic factors [100, 101]. These factors not only remodel ECM, but also promote angiogenesis and control collagen fibers formation.

In passive intravasation, tumors spread through inactive shedding [96]. It has been shown that large numbers of space-deprived tumor cells push against each other, causing stress which might compress the vessels and as a consequence, drive tumor cells into the vessels [102]. Another study supporting passive intravasation demonstrated that primary tumor trauma or massage increases the amount of cancer cells entering the bloodstream [103].

Migratory properties, which enable tumor cell intravasation, may be acquired during a process called epithelial-mesenchymal transition (EMT), which is characterized by loss of cell adhesion, repression of E-cadherin expression, as well as increased cell motility. Tumor cells, which undergo EMT may either intravasate as single cells or cooperate with non-EMT cells, helping them to complete the spontaneous metastasis process. However, even though both cell types persist in the circulation, only the ones with adhesive properties are able to attach to the vessel wall and extravasate into the secondary metastasis site [95]. Mesenchymal phenotype of an EMT cell may be reverted by mesenchymal-epithelial transition (MET), which means that the

same cell is able to first enter into the vessel due to EMT and subsequently undergo MET, extravasate and continue metastatic process.

1.2.3.2.2 Lymph nodes infiltration

In the second hypothesis concerning metastasis formation, cancer cells initially invade lymph nodes and subsequently spread from lymph nodes to the blood vessels. This might be possible by tumor cell migration to the efferent lymphatics, followed by reaching vena cava, enabling further hematogenous dissemination. Although lymphatic intravasation is strongly associated with tumor lymphangiogenesis, mechanisms linking lymphangiogenesis with cancer metastasis are still under discussion. In one of the studies, PDGF-BB expression in murine fibrosarcoma cells induced tumor lymphangiogenesis, increasing number of lymph node metastases [104]. In other studies, tumor cells have been shown to invade into lymphatics and form metastasis through the expression of chemokine receptors, such as CXCR4 and CCR7, corresponding to expression of respective ligands (CXCL12 and CCL21) in secondary metastatic sites [105].

1.2.3.3 Circulating tumor cells

Cells that have detached from the primary tumor and successfully enter the bloodstream are referred to as circulating tumor cells (CTCs). Their presence and behavior provide information on the biology of cancer and factors such as structure of vessels and metastatic potential of tumor. Breast cancer patients with higher levels of CTCs as compared with the group having lower CTCs levels, had a shorter median progression-free survival and shorter overall survival, what led to the conclusion that number of CTCs is an independent prognostic factor for patients survival at any time of breast cancer disease [106].

The role of CTCs as a prognostic factor has been also demonstrated in castration-resistant prostate cancer patients [107], as well as metastatic breast and colon cancer patients [108, 109] with use of CellSearch system developed by Veridex, a method approved by Food and Drug Administration (FDA) for the detection of CTCs in whole blood. CellSearch technology is based on immunofluorescent labeling of epithelial cells (CD45-, EpCAM+, and cytokeratins 8, 18+, and/or 19+) combined with automatic confocal microscopy. Despite its advantages, the method is expensive, not sensitive enough and detects only CTCs with epithelial phenotype. Since it is known that aberrant activation of EMT process occurs during tumor progression [110], CTCs can also exhibit mesenchymal phenotype [111]. Thus, the dynamic changes between epithelial and mesenchymal phenotypes of CTCs have to be further investigated in order to understand the role of specific CTCs subsets in cancer metastasis.

1.2.3.4 Direction of metastatic spread

Occurrence of metastases is the one of the most intriguing, yet still not enough explored areas in cancer field. The distribution pattern of secondary growths in breast cancer has been originally proposed by Paget et al. [112], according to ‘seed and soil’ hypothesis. This hypothesis assumes that cancer cells, in order to metastasize, have to find a location with similar characteristics to their original microenvironment [113]. Nevertheless, according to another theory, proposed by James Ewing, metastasis occurs only through anatomic and mechanical routes.

It is known that particular tumor types infiltrate certain organs but the detailed explanation of this phenomenon is still unknown. However, identification of genetic determinants of cancer metastasis [114] and CTCs' infiltration into specific organs [115, 116] provided new insights into targeted cancer spread.

Breast cancer metastasizes mainly to bone, lungs, regional lymph nodes, liver and brain. There are many various barriers that prevent CTCs to infiltrate specific organs, including basement membrane as a physical barrier, reactive oxygen species, low pH and hypoxia as chemical barriers and many biological factors, such as immune response or cytokines [117]. Moreover, organ-specific metastasis is largely influenced by expression of receptors on tumor cells, regulation by adhesion molecules and chemoattractants, as well as certain physical features of colonized organs. For example, bone marrow, as one of most common secondary sites of metastasis, is built of capillaries, referred to as sinusoids, which are composed of epithelial cells cross-linked with cell gates allowing for penetration of hematopoietic cells [118]. In a similar manner, the structure of liver capillaries facilitates CTCs' extravasation [119]. In lung vasculature, which is another frequent metastasis target site, a metadherin binding site has been found, a protein that is overexpressed in metastatic breast cancer [120]. Compared to the other metastatic sites, brain is the most difficult organ to colonize, as cancer cells have to pass the blood-brain barrier in order to create micrometastases. However, the homing or adhesion mechanisms, facilitating brain metastasis still remain unclear.

1.2.4 Cancer immunology

Cancer immunology is an area in medicine and research, focused on the interplay between cancer and immunological system. There are two main concepts which are closely related to cancer immunology – immunosurveillance and immunoediting. The idea that the immune system could defend the host from neoplastic disease was originally proposed by Ehrlich [121] in 1909, but officially cancer immunosurveillance term was introduced nearly 50 years later by Burnet and Thomas, who identified lymphocytes as main guardians in immune sytem that recognize and destroy quickly arising transformed cells [122]. Described process aims at host protection, inhibition of cancer development and maintenance of cell homeostasis [123]. Immunosurveillance is included into more basic concept of immunoediting, which assumes that immune system is a major host defender against cancer [123]. Immunoediting involves three consecutive stages: elimination, equilibrium and escape [124].

Elimination stage consists of a series of events, initiated when the innate immune system cells recognizes the presence of a growing tumor, due to local tissue disruption, caused by stromal remodeling process. This remodeling process, resulting from angiogenesis and tissue-invasive growth, induces production of inflammatory molecules, which together with tumor-produced chemokines [125] recruit the innate immune system cells to the tumor site. Among recruited cells there are natural killers T (NKT) cells, natural killer (NK) cells, macrophages and dendritic cells, which use different means of ligand recognition of the tumor cells. This process leads to the production of IFN- γ, which is a key step in progression of antitumoral response. Subsequently, IFN- γ induces local production of chemokines, which recruit more cells of the innate immune system, and lead to tumor death by activating several IFN-γ-mediated processes, having an angiostatic [126], proapoptotic [127] and antiproliferative [128] effects on the tumor.

Additionally, IFN- γ-activated macrophages and NK cells can destroy tumor cells by either perforin [129], or TRAIL-dependent [130] mechanisms. Consequently, tumor antigens become accessible and tumor-specific adaptive immune system becomes involved. Immature dendritic cells that have been attracted to the tumor area are activated via cytokines released by innate immune response or interaction with recruited NK cells [131]. After activation, dendritic cells acquire tumor antigens and migrate to the draining lymph nodes [132], where they trigger differentiation of naïve tumor-specific Th1 cells. Th1 cells are a subgroup of CD4+ T-cells, which facilitate development of tumor-specific CD8+ cytotoxic T cells [133]. Finally, CD4+ and CD8+ T-cells home to the tumor site and kill the antigen-bearing tumor cells.

Cancer cells that have not been eradicated during the previous phase enter equilibrium stage. In this stage, IFN- γ and lymphocytes exert selection pressure on remaining tumor cells, which lack genetic stability. Even though many tumor cells are destroyed during this process, new tumor cells clones with decreased immunogenicity arise.

Selected tumor clones enter escape stage and are able to expand ad form clinically detectable tumors. Cancer cells use different strategies to avoid host's antitumor immune response, such as release of immunosuppressive cytokines (IL-10, TGF-β) or involvement of regulatory T cells [124]. Tumor escape might also be a result of changes occurring directly at the tumor level. These changes might affect tumor recognition by effector cells, for example by losing MHC components [134], losing tumor-specific antigen expression, downregulation of NKG2D [135] or developing insensitivity to IFN- γ [136]. Other possible alterations allow the tumors to escape immune responses, for example by developing apoptosis resistance [137] or faulty death-receptor signaling [138]. Identification of more escape mechanisms might provide essential information on interactions between the tumor and immune system.

Cancer immunology research is focused on discovering new cancer immunotherapies, such as vaccines or antibody therapies. In order to achieve this, it is necessary to identify the targets for immune recognition in cancer [122]. For example, presence of lymphocytic infiltration in the tumor have been correlated with improved clinical outcomes of colorectal cancer patients [139].

1.2.5 C/EBPβ

C/EBPβ is a protein belonging to C/EBP (CCAT-enhancer-binding protein) family, which regulates expression of several genes controlling cell proliferation, differentiation, inflammation and metabolism. C/EBP family consists of six transcription factors called C/EBPα to C/EBPζ and is characterized by presence of basic leucine zipper (bZIP) domain at the C-terminus, which is required for dimerization and DNA binding.

C/EBPβ can form heterodimers with other members of the C/EBP family, such as C/EBPα, C/EBPγ and C/EBPδ, as well as with other transcription factors like CREB1 [140] or Sp1 [141]. It is also capable of binding as a homodimer to certain DNA regulatory regions and hence, controlling the expression of numerous target genes. Among them, there are genes playing a role in the nervous system, such as PPT-A (preprotachykinin) gene [142] and the choline acetyltransferase gene [143].

The intronless C/EBPβ gene encodes three different protein isoforms: LAP1, LAP2 (liver-enriched activator proteins) and LIP (liver-enriched inhibitor protein), expressed depending on differential use of specific in-frame translation sites [144, 145]. While LAP1 and LAP2 are transcriptional activators, LIP usually plays the role of their inhibitor. Nevertheless, functions of all C/EBPβ isoforms are still under investigation and largely dependent on the cellular context. Alternative C/EBPβ isoform production is tightly regulated by the mammalian target of rapamycin (mTOR) pathway, where mTOR activation stimulates LIP expression and mTOR inhibition enhances LAP production [146]. Additionally, the truncated LIP isoform may beformed by partial proteolysis of longer C/EBPβ isoforms [147]. While all the isoforms contain C-terminal bZIP domain, LAP1 and LAP2 harbor also N-terminal transactivation and chromatin remodeling domains. The ratio of C/EBPβ isoforms is thought to determine cell fate [148, 149].

As a transcription factor, C/EBPβ interacts with several target genes and is required for a variety of biological processes, such as adipogenesis (126-129), embryogenesis [150], granulopoiesis [151], muscle repair (116-118) and osteoporosis (121-125). Moreover, it is involved in controlling cell growth, autophagy and antibacterial defense (130-131), as well as in regulating insulin level and insulin receptors expression (132-134). Finally, C/EBPβ regulates multiple genes responsible for immune and inflammatory responses. It has been shown to bind to cytokine coding genes such as IL-6 [152], IL-4 [153], IL-5 [154] and TNFα [155] and be responsible for activation and terminal differentiation of macrophages, an important immune cell subtype (75). As C/EBPβ expression plays a role in astrocyte inflammatory response, its expression is changed in various neurological disorders including Alzheimer's disease [156], Parkinson's disease [157] and HIV-1-associated dementia [158].

In this thesis, particular emphasis is put on the role of C/EBPβ in breast cancer. Its gene is usually not mutated in this form of cancer. A few rare mutations that have been found are doubted to contribute to epithelial tumors [159]. Nevertheless, C/EBPβ might be amplified in a small subgroup of breast neoplasia, described as lobular carcinoma in situ [160]. Elevated levels of C/EBPβ mRNA are associated with metastatic breast cancer [161], higher tumor grade [162, 163] and overall worse prognosis [161].

1.2.5.1 Mammary gland development

Both LAP1 and LAP2 are expressed in non-malignant, human mammary epithelial cells [164], as well as in breast tumors [165, 166]. LAP1 is mainly expressed in normal mammary cells, whereas LAP2 is found only in dividing cells, both normal and neoplastic [167].

Studies *in vitro* as well as *in vivo* have showed significant contribution of C/EBPβ isoforms to the mammary gland development and breast cancer. For instance, *in vitro* studies have shown that LIP overexpression in mouse mammary epithelial cells [168] or fibroblasts [169] causes lack of contact inhibition, increased proliferation and foci formation. Additionally, transgenic mice with overexpression of LIP in the mammary gland develop alveolar hyperplasia, high grade mammary intraepithelial neoplasias as well as invasive and noninvasive carcinomas [168].

Other studies, using C/EBPβ-/- mice, revealed that C/EBPβ is necessary for ductal morphogenesis, epithelial cell proliferation and functional differentiation in the murine mammary gland [170, 171]. However, mice with abolished LAP2 expression displayed no abnormalities in

mammary gland development [172], what suggests that expression of this isoform is not crucial in this process and that LAP1 and LIP might compensate for the loss of LAP2.

1.2.5.2 Cell survival and apoptosis

The role C/EBPβ in cancer might be also explained by its ability to regulate cell survival and apoptosis. For example, C/EBPβ is essential for oncogenic transformation of macrophages and controls expression of insulin-like growth factor I (IGF-I), which is an important prosurvival factor [173]. C/EBPβ is also critical in survival of hepatic stellate cells bearing DNA damage due to CCL_4-induced free-radical formation [174]. Additionally, C/EBPβ promotes cell survival by repression of p53 expression and altering its function in keratinocytes in response to DNA damage [175] and carcinogen-induced stress [176].

Apoptosis is a process of programmed cell death controlled by various factors. It has been demonstrated that MAPK-RSK-C/EBPβ signaling is crucial for liver fibrosis and modulates stellate cell survival through caspase 8-associated protein FLIPL [177]. In addition, studies on C/EBPβ-null mice display lowered apoptotic liver injury and reduced activation of caspase-3 [178]. Reduced levels of C/EBPβ may also lead to resistance to DMBA-induced skin tumorigenesis by increased apoptosis [179].

1.2.5.3 Cell growth and senescence

C/EBPβ is often involved in proliferation, for example in liver, where this process is promoted via interaction of C/EBPβ with HDAC1 [180]. On the other hand, *in vitro* studies have shown that forced expression of LAP2 may lead to growth arrest in normal cells, such as keratinocytes [181] or fibroblasts [182], as well as in cancer cells like hepatocarcinoma cells [183]. It has also been demonstrated that oncogene-induced senescence involves C/EBPβ-dependent expression of certain chemokines, such as CXCR2 [184] and activation of the inflammatory networks [185]. In addition to that, C/EBPβ is required for Ras-induced senescence by acting downstream or independently of p19ARF/p53 [186].

Overexpression of LAP2 in MCF10A cells leads to foci formation, anchorage independence, epithelial-mesenchymal transition and acquirement of invasive phenotype [187]. Nevertheless, LAP2 overexpression in LIP-rich breast cancer cells causes decreased proliferation and senescence [166]. Hence, the ratio of C/EBPβ isoforms is essential for normal cell growth and development and their altered expression may lead to aggressive forms of breast cancer. For example, a high LIP: LAP ratio in human breast carcinomas correlates with more aggressive phenotype and poor prognosis [165, 188, 189].

1.2.5.4 Epithelial-mesenchymal transition (EMT)

Epithelial-mesenchymal transition (EMT) is a reversible process allowing epithelial cells to lose their cell polarity and cell-cell adhesion, and to achieve migratory and invasive abilities to trans-differentiate into mesenchymal-like cells [190, 191]. It has been shown that cycles of both EMT and MET (mesenchymal-epithelial transition) are required for embryo formation [192-194]. Nevertheless, EMT occurs also in wound healing, organ fibrosis, as well as cancer progression [192, 195].

Epithelial cells are in close contact to each other owing to tight junctions, gap junctions and adherens junctions, have apico-basal polarity high E-cadherin expression, polarized actin cytoskeleton and rest on a basal lamina. Conversely, mesenchymal stem cells communicate with each other only by focal points [196], lack polarization and display spindle-shaped morphology, expressing factors such as N-cadherin, fibronectin and vimentin. Therefore, EMT involves prominent changes on a morphological, as well as phenotypical level.

The crucial event in EMT involves loss of E-cadherin, which is controlled by different transcription factors. While Snail1, Snail2 (Slug), ZEB1, ZEB2, E47 and KLF8 repress E-cadherin transcription, other factors like Twist, Goosecoid, TCF4 or FOXC2 repress E-cadherin expression in an indirect manner. In addition, EMT can be induced by several growth factors in cancer, including Notch [197], epidermal growth factor (EGF) [198], hepatic growth factor (HGF) [199], Wnt factors [200], and transforming growth factor β (TGF-β) [201], which is considered to be a major EMT inducer in cancer.

Studies on the invasive cancer front showed cancer cells expressing both epithelial and mesenchymal markers, spreading from tumor mass into the adjacent stroma. Different cancer cell phenotypes and EMT were described in multiple cancer types, including breast [202], colon [203], cervical [204], thyroid [205], ovarian [206] and oral squamous cell carcinoma [207]. Moreover, EMT have been shown to promote metastasis [208] and associated with metaplastic [209] and basal-like breast carcinoma [210], as well as other higher tumor grades, what suggests that EMT might aggrevate clinical outcome [211]. Additionally, some of EMT markers, like Snail and Twist, correlate correlate with relapse of breast [212], bladder [213] and ovarian cancer [214].

1.2.5.5 Associations with breast cancer treatment

Interplay between epithelium and stroma is crucial for aromatase expression and estrogen production in breast cancer tissue [215]. Upregulated aromatase expression in breast fibroblasts increases production of estradiol (E2), activating several carcinogenic genes via estrogen receptor-α (ERα) in malignant epithelial cells. In order to avoid this effect, aromatase inhibitors (AIs) are used in the clinics, being nowadays the most effective hormonal treatment for estrogen-responsive breast cancer [216]. As aromatase expression is controlled by different signaling pathways, it is highly desirable to target these pathways and inhibit estrogen production in selected tissues. C/EBPβ has been shown to regulate aromatase expression in human fibroblasts in the breast adipose tissue, and therefore, it might indirectly contribute to breast cancer progression [217].

Another example of indirect role of C/EBPβ in breast cancer is its ability to activate the genes conferring multidrug resistance to the cells, such as ABCC2 [218] or ABCB1 [219-221]. These genes encode proteins belonging to ATP-binding cassette transporters (ABC-transporters) family, which are frequently overexpressed in breast cancers. Moreover, elevated levels of ABC transporters like ABCB1 have been correlated with chemotherapeutic outcomes [222], what suggests that C/EBPβ, by activation of ABCB1, plays a secondary role in chemoresistance in breast cancer.

Receptor tyrosine kinase signaling has a significant function in development of various types of cancer. Mammary tumorigenesis in particular is influenced by epidermal growth factor (EGF)

receptor and fibroblast growth factor (FGF) receptor families, which are strongly associated with C/EBPβ [223]. For example, it has been demonstrated that ErbB1–EGFR signaling regulates the differential translation of the LAP and LIP isoforms, causing increased LIP expression [164, 224] and subsequently, higher LIP:LAP ratio, which contributes to promoting proliferation and a more aggressive disease phenotype. In addition, C/EBPβ is involved in EGFR regulation of FGF activity and FGFR2 expression in breast epithelial cells [225, 226].

C/EBPβ has been also proven to negatively regulate one of the microRNAs that is often downregulated in tumors, miR-145. Both isoforms of C/EBPβ take part in miR-145 regulatory system involving Akt pathway, what might explain the downregulation of miR-145 in cancer cells [227].

In summary, C/EBPβ is involved in many aspects of breast cancer development and progression by affecting several biological pathways. However, additional *in vitro*, as well as *in vivo* studies are needed to unravel the specific mechanisms explaining C/EBPβ function in breast cancer.

1.2.6 MSCs and chemokines in cancer

Mesenchymal stem cells as a part of tumor microenvironment are important factor affecting tumor development [228]. However, their role in this process still remains controversial. Dependent on the biological context and multiple factors, they might either promote tumor progression or act as tumor suppressors. Using different sources of tissue, individual donor variability and timing of MSCs injection contributes to discrepancies between the studies. Furthermore, expression of certain receptors (e.g. toll-like receptor, TLR) fluctuates in different time points during MSCs treatment [229], what may also affect MSCs influence on cancer progression. Although it is clear that MSCs administered systemically migrate towards tumors [230, 231], their precise role in cancer development is still under discussion.

MSCs have been shown to have therapeutic potential in targeting several cancer types, including breast cancer [232], brain tumors [233], liver cancer [234, 235], leukemia [236], pancreatic cancer [237] and sarcoma [238] in various animal models. Some reports indicate that MSCs suppress tumor development by cell fusion [239] or through p38 mitogen-activated protein kinase (MAPK) [240].

Conversely, other studies revealed that MSCs support tumor growth in colon cancer [241], lymphoma [242] and melanoma [243]. However, this effect was explained by Klopp *at al.*, who suggested that increased MSCs proliferation in the tumor leads to augmented tumor mass [244]. Moreover, MSCs in tumor stroma were shown to promote breast cancer metastasis by immune modulation [245] and regulate EMT and tumor progression in pancreatic cancer cells [246]. Interestingly, MSCs have also been reported to be a source of cancer-associated fibroblasts (CAFs) which are major contributors to cancer progression [247].

There are numerous reports describing the function of MSCs in colon cancer progression. Specifically, MSCs modulate the tumorigenicity of human colon cancer cells via interleukin-6 (IL-6) [248] and promote formation of murine colorectal tumors [249]. Moreover, MSCs enhance growth and metastasis of colon cancer by promoting angiogenesis, migration, and invasion and by repressing tumor cell apoptosis [241].

Homing of MSCs to the tumor involves action of multiple factors, controlling cell migration towards inflammation, such as growth factors, chemokines and cytokines. One of the chemokines engaged in this process is stromal cell derived factor 1α (SDF1α), which secreted from cancer cells enhances MSCs migration towards the tumor in Helicobacter-mediated gastric cancer model [250]. Another example is chemokine (C-C motif) ligand 5 (CCL5), which augments cancer cell migration and invasion, and thereby, contributes to MSC-induced breast cancer metastasis [245].

In addition to chemokines, chemokine receptors affect tumor progression as well. Namely, CCR1 was reported to mediate accumulation of myeloid cells in the liver microenvironment, enhancing mouse colon cancer metastasis [251, 252]. Furthermore, CCR1 promotes liver metastasis by regulating angiogenesis [253]. Another chemokine receptor, CCR2, has been found to be upregulated in MSCs subject to irradiated tumor cells and its inhibition resulted in diminished MSC migration *in vitro* [254].

1.2.6.1 CCL7 in cancer

Chemokine (C-C motif) ligand 7 (CCL7), known also as monocyte-specific chemokine 3 (MCP-3), is a chemokine which attracts monocytes and regulates function of macrophages. Identified originally from osteosarcoma supernatant [255], CCL7 expression was observed in several cell types, including monocytes, fibroblasts, platelets, colon epithelial cells and some tumor cell lines [256-258]. As CCL7 interacts with multiple leukocyte receptors, it is capable of activating monocytes and granulocytes [259]. In support of this, CCL7 has been also shown to drive TNFα-dependent Th1/Th17-mediated inflammation in psoriasis. Finally, locally overexpressed MCP-3 was shown to recruit MSCs to injured tissue and improve cardiac remodeling [260].

The topic of CCL7 role in tumor progression appeared in the literature already over a decade ago. One of the first described studies indicated that CCL7 gene transfer to mastocytoma cells resulted in reduced tumorigenicity, increased neutrophil recruitment to the tumor and enhanced dendritic cell infiltration in peritumoral tissue [261]. Another study showed that CCL7 gene transfection to colorectal cancer cell line resulted in tumor growth retardation and metastasis inhibition [262]. On the other hand, several other research groups revealed that CCL7 expression can be associated with enhanced invasive and metastatic properties. Namely, CCL7 was reported upregulated in cancer-associated fibroblasts (CAFs) cultured with oral squamous cell carcinoma (OSCC) cells and promoted tumor migration and invasion *in vitro* [263]. Subsequently, CCL7 overexpression was associated with lymph node metastasis and poor prognosis in gastric cancer [264]. Moreover, brain cancer metastases were shown to carry higher expression of CCL7 compared to primary tumors of renal cell cancer (RCC) [265]. Finally, recent studies confirmed the role of COX2-MMP1/CCL7 axis in brain cancer metastasis [266].

Interestingly enough, CCL7 receptors CCR1, CCR2 and CCR3 were found overexpressed in liver metastatic tumor tissues of the patients and had upregulated expression levels in hepatic recurrences, when compared with primary tumors [267]. It was also the first clinical report presenting CCL7 as a novel target in liver metastasis of colorectal cancer. Nevertheless, the mechanisms underlying CCL7 importance in colorectal metastasis have not been yet explained.

2 AIMS OF THE STUDIES

- quantitative and functional characteristics of MSCs and pericytes in the vascular wall
- characterisation of receptors for chemokines, cytokines and growth factors present on MSCs and pericytes and determining how chemokines and cytokines modulate migration and differentiation of mural cells
- studying the relationship between EMT and C/EBPβ expression in breast cancer
- analyzing function of C/EBPβ in formation of mestastasis and its effect on vascular morphology
- determination if C/EBPβ can be one of the prognostic markers in diagnostics of metastatic breast cancer patients
- analyzing crosstalk between MSCs and colon cancer
- discovering the role of CCL7 in colon cancer progression

3 RESULTS AND DISCUSSION

3.1 STUDY I

Intimal hyperplasia is not only a healing response, but also main cause of vessel narrowing after injury. This process involves migration of vascular progenitor cells, what might lead to transplant arteriosclerosis. The goal of this study was to analyze the role of MSCs in intimal hyperplasia and to determine factors affecting their recruitment. Rejection of transplanted organs is often associated with inflammatory response and secretion of factors activating cells in surrounding tissues. Therefore, in order to find out how allografts influence the adjacent vessel tissues, we transplanted a fragment of rat isogenic (from F344 rats) or allogenic (from LEW rats) aortic graft into the abdominal aorta of F344 rats and collected samples of tissues at different timepoints.

Immunohistohemical analysis of 2-week-old allografts showed decreasing α-smooth muscle actin expression in medial layer and migration of α-smooth muscle actin positive cells to neointima. Adjacent vessels displayed similar, but less extensive structural alterations. Interestingly, electron microscopy performed at 1 to 2 weeks after transplantation, showed architectural changes in SMCs in the inner part of the media and progressing allograft de-endothelization. While most of SMCs had a contractile phenotype with well-organized myofilaments, some of them exhibited synthetic phenotype characterized by increased Golgi system and ER size. Once again, similar but less prominent changes were observed in the adjacent vessels.

Staining for α-smooth muscle actin at week 4 showed less SMCs in the media, coinciding with increasing fibrosis and inflammation. In the neointima, we observed more SMCs and inflammatory cells.

Remodeling process continued until week 8, when inflammation in the allograft had already started decreasing. Medial layer developed fibrosis with enhanced ECM and allograft intima grew in size, mainly consisting of contractile SMCs and leukocytes. On the contrary, adventitia did not show structural abnormalities. The study revealed that adjacent tissue underwent structural alterations similar to the ones in allograft, but less prominent. Namely, in adjacent tissue, a thin layer of intima, as well as SMCs of both contractile and synthetic phenotypes in the media were observed.

In order to assess if neointimal cells may be derived from other vessel layers in the allograft or adjacent vessels, we performed transplantation of female-to-male F344 isotypic insert into a female LEW rat. Real time PCR analysis for SRY gene of aortic allograft intimal lesion revealed that cells derived from adjacent vessel were detected at low levels in the allograft, only at late timepoints (12 weeks after transplantation) and in 25% of rats. Therefore, possibility of adjacent vessel being a source of migratory cells contributing to formation of allograft intima was excluded.

Subsequently, another experiment was performed to determine if adventitia could provide the cells incorporated into intima. Specifically, orange cell tracer labeled male LEW adventitia was transplanted into F344 female allografts that had previously been transplanted into LEW rats. In this setting, the isogenic labeled adventitia was not subject to allogenic

inflammation. 14 days after transplantation, we observed extensive migration of labeled cells into the intima, where nearly 80% of intimal cells originated from adventitia. To determine different subpopulations of adventitial progenitor cells migrating into the intima, we used flow cytometry analysis, which identified CD90+, CD44+ and sca-1+ cells as predominant cell phenotype in the adventitia. Immunohistochemical analysis and confocal microscopy revealed equal distribution of described cell populations in the adventitia and only a few CD90+, CD44+ and sca-1+ cells in the media. Double stainings for the previously used progenitor cell markers were performed and indicated that nearly 14% of adventitial cells were double-positive for CD90 and CD44, while only 3-6% of cells were double-positive for sca-1 and CD90 or CD44.

Next, RNA microarray using allografts and adjacent vessel 2 and 8 weeks after transplantation was used as a screening method for essential factors for the recruitment of adventitia-derived cells to the intima. Increased expression of chemokines such as MCP1-1, RANTES, SDF-1 and interferon-inducible protein-10, as well as their receptors like CCR2, CCR5, CXCR3 and CXCR4, was found and confirmed by real time PCR. Additionally, PCR assays performed on nontransplanted aortas confirmed high levels of expression of mentioned chemokine receptors in adventitial cells.

Afterwards, migration assays on rat adventitial cells were performed in response to MCP-1, RANTES, SDF-1 and interferon-inducible protein-10, which identified MCP-1 as the most potent chemoattractant. Furthermore, double stainings for CCR2 (MCP-1 receptor) and progenitor cell markers used for flow cytometry analysis confirmed expression of CCR2 on CD90+, CD44+, sca-1+ cells, and to smaller extent also on NG2+ cells.

We followed our study using injury model as a way to determine if MCP-1 contributes to formation of intimal hyperplasia in a nontransplant model. Namely, arteries derived from C57BL/6 and MCP-1 knockout mice were mechanically injured and followed by transplantation of the labeled adventitia from C57BL/6 and CCR2 knockout mice. Almost half of the adventitial cells migrated to the intima and formed intimal lesion. Moreover, both transplantation to MCP-1 knockout mice and transplantation from CCR2 knockout mice hindered migration of adventitial cells by approximately 25%.

Fluorescent immunohistochemical analysis for cells coexpressing CCR2 and CD90, CD44 and sca-1 demonstrated presence of double-positive cells mainly in the adventitia and in the outer area of the media, what provided further proof of presence of MSCs in the vessels and their role in intimal hyperplasia.

Subsequently, we showed that most of the MSCs derived from adventitia were able to differentiate into SMCs. Specifically, we sorted CD90+, CD44+, sca-1+ and NG2+ cells using flow cytometry, cultured them with PDGF-BB to induce SMC differentiation and stained them for α-smooth muscle actin and myosin.

Finally, a fusion assay was performed between SMCs and adventitial cells by means of creLOX system in order to exclude the probability that adventitia-derived intimal SMCs are adventitial progenitor cells that have fused with SMCs. As a result, no fusion was noticed between adventitial cells and SMCs, even under forced conditions.

This study identified mesenchymal stem cells of adventitial origin as a cellular source contributing to intimal hyperplasia. Furthermore, it recognized MCP-1 as a potent chemokine involved in recruitment of adventitial progenitor cells to intimal lesions.

3.2 STUDY II

Cancer progression involves multiple factors, one of which is TGFβ. During this process, TGFβ role switches from growth inhibitor at early stage to a major inducer of EMT, invasion and metastasis in later stage. C/EBPβ, an essential factor regulating mammary gland development, has recently been shown to be deregulated in breast cancer [223]. Additionally, studies published by Bundy *et al.* suggested that C/EBPβ might be involved in the TGF-β response towards both growth inhibition and EMT [187]. Therefore, we decided to determine if loss of C/EBPβ has a preventive role against TGF-β- induced EMT in breast cancer.

In order to examine the relationship between EMT and C/EBPβ expression in breast cancer cells, immunofluorescent staining followed by confocal microscopy analysis of samples of human breast carcinoma was performed. Specifically, C/EBPβ was stained in combination with E-cadherin, which is an EMT marker, commonly lost in invasive cancer. In samples of well-differentiated DCIS, we observed prominent E-cadherin expression at cell-cell junctions and nuclear expression of C/EBPβ in breast cancer cells.

In case of invasive ductal breast carcinomas, previously classified according to their estrogen, progesterone and HER2 receptor status, we found decreased E-cadherin expression in areas of triple-negative tumors. Moreover, these tumors expressed lower levels of E-cadherin compared with ER+, PR+ and HER2+ tumors and with HER2+ tumors. Nuclear C/EBPβ staining was diminished in triple-negative tumors and linear regression analysis revealed significant correlation between expression of E-cadherin and C/EBPβ in all analyzed IDC samples. Further examinations indicated to decreased C/EBPβ expression in E-cadherin-negative areas compared with E-cadherin-positive areas of the triple-negative tumors.

In order to analyze C/EBPβ expression during breast cancer progression, we used transgenic mouse model overexpressing the polyoma virus middle T antigen under mouse mammary tumor virus promoter (MMTV-PyMT mice). Fluorescent double stainings of tumors derived from 10- and 14- week old MMTV-PyMT mice showed prominent loss of C/EBPβ expression in E-cadherin-negative areas compared with E-cadherin-positive areas of adenocarcinomas.

To study if C/EBPβ is regulated during EMT, we employed commonly used model of TGF-β1-induced EMT in mouse mammary gland epithelial cells (NMuMG). NMuMG cells, after 48h treatment with TGF-β1, had not only decreased nuclear expression of C/EBPβ, but also showed typical signs of EMT, such as: exhibited elongated phenotype, lost expression of E-cadherin and upregulated expression of vimentin. Additionally, Western blot analyses confirmed EMT phenotype of TGF-β1-treated NMuMG cells, showing decreased levels of the junction proteins E-cadherin, CAR, occludin and claudin-3, and enhanced levels of N-cadherin and vimentin, compared with untreated cells. All isoforms of C/EBPβ (LAP1, LAP2 and LAP) were decreased in examined cells and no changes in LIP/LAP ratio were observed. Furthermore, loss of C/EBPβ was shown to be specifically associated with EMT. Namely, EpH4 cells, which are resistant to TGF-β1-induced EMT were also treated with TGF-β1. As a result, neither morphological signs of EMT, nor changes in the expression of junction proteins

or mesenchymal phenotype proteins, such as N-cadherin or vimentin, were revealed. Additionally, TGF-β1 treatment caused activation of Smad3, but did not affect C/EBPβ levels.

qPCR analyses showed that TGF-β1 treatment in NMUMG cells reduced mRNA level of E-cadherin (*Cdh1*) by 60%, CAR (*Cxadr*) by 40% and C/EBPβ (*Cebpb*) only by 20%, what suggested possible involvement of posttranscriptional mechanisms in repression of C/EBPβ during TGF-β1-induced EMT. In order to verify if microRNA-155 (miR-155), a well-recognized oncomiR in breast cancer [268], which targets *Cebpb* in macrophages and B cells [269, 270], takes part in repressing C/EBPβ in TGF-β1-dependent EMT, NMuMG cells were treated with miR-155 inhibitor before TGF-β1 treatment. Inhibitor increased C/EBPβ expression at baseline and decreased its repression during EMT, what was confirmed by immunofluorescence analysis. Interestingly, additional inhibitor's effect of 20% rescue of the repression of E-cadherin was confirmed only by qPCR, but not by immunofluorescence or immunoblotting. On the other hand, using miR-155 mimic instead of miR-155 inhibitor caused diminished levels of C/EBPβ at baseline, and further repression after TGF-β1 exposure. Further qPCR comparisons between the cells indicated that miR-155 levels were approximately 4.5 fold higher in NMuMG cells compared with EpH4 cells at baseline, and even more increased in NMuMG cells upon TGF-β1 treatment, as shown in previous studies [271]. Moreover, all three isoforms of C/EBPβ had higher expression in EpH4 cells compared with NMuMG cells. Loss of C/EBPβ during TGF-β1-induced EMT was inhibited by both siRNA against Smad3 and commercially available inhibitor, what proved that repression of C/EBPβ was mediated through the canonical TGF-β/Smad3 pathway.

To determine whether miR-155-dependent loss of C/EBPβ might sensitize cells to TGF-β1-induced EMT, we performed loss- and gain-of-function studies for C/EBPβ. Lentivirus-mediated knockdown of C/EBPβ through overexpression of small hairpin RNA (shRNA, in NMuMG cells led to decreased expression of E-cadherin and CAR at baseline, as well as potentiated EMT induction after exposure to TGF-β1, demonstrated by further decrease of expression of E-cadherin and CAR and increase of expression of vimentin compared with cells expressing control shRNA. Conversely, transient overexpression of LAP2 in NMuMG cells caused increased mRNA levels of *Cdh1* and *Cxadr* at baseline and diminished repression of those genes after treatment with TGF-β1.

Subsequently, to assess if C/EBPβ re-expresssion would suffice to revert cells from a more established EMT phenotype, we used long-term EMT induction in NMuMG cells, known to cause prominent EMT and evasion of cytostatic effects of TGF-β1. NMuMG, treated for 14 days with with TGF-β1 developed profound EMT phenotype with low expresion of E-cadherin, CAR and C/EBPβ. LAP2 overexpression in long-term treated NMuMG cells led to increased expression of E-cadherin and CAR, decreased expression of vimentin, but not to EMT phenotype reversion as observed morphologically. Since overexpression of LAP2 did not change expression of Zeb1, Snail, Slug and Twist, we assessed that induction of E-cadherin and CAR was not dependent on expression of those EMT factors. As already shown in previous studies, long-term treated NMuMG cells evaded growth inhibitory effect of TGF-β1 and proliferated in a similar way to untreated cells. Additionally, LAP2 overexpression sufficed to restore the growth inhibitory effect of TGF-β1 in studied cells.

To study the effect of C/EBPβ expression on invasion, we performed chemoinvasion assays on NMuMG cells subject to TGF-β1. NMuMG cells expressing *Cebpb* shRNA were 60% more invasive than cells expressing control shRNA, while NMuMG cells overexpressing LAP2 were 40% less invasive compared with control cells.

Since invasive properties are closely related to metastasis, we decided to determine if loss of C/EBPβ would influence metastatic capacity of breast cancer cells *in vivo*. Loss-of-function study in 4T1 breast cancer model, driven by TGF-β, resulted in lower E-cadherin expression, higher vimentin expression and enhanced invasiveness towards TGF-β1. Tumors of 4T1-implanted mice grew initially at similar pace to control tumors but their growth rate decreased during second week after implantation. Average metastatic foci number in lungs was higher in mice carrying C/EBPβ knockdown tumors compared with mice carrying control tumors. Moreover, metastasis was found in 100% of mice with C/EBPβ knockdown vs. in only 20% of control mice.

In order to verify the hypothesis that C/EBPβ could regulate the EMT response downstream of TGF-β by serving as a transcriptional activator of junction proteins, we firstly used computer-based software enabling us to find binding sites for C/EBP transcription factors in genomic DNA sequences. Analysis revealed such putative binding sites in promoter regions of genes encoding junction proteins such as *Cdh1* and *Cxadr*. ChIP assays confirmed specific interactions between C/EBPβ and regions of *Cdh1* and *Cxadr* promoters containing C/EBP binding sites in NMuMG cells. We showed that C/EBPβ overexpression activated *Cdh1* promoter by twofold and the *Cxadr* promoter by 2.5-fold. While LAP2 was more potent compared with C/EBPβ, by activating mentioned promoters by 5- and 4-fold, respectively, LIP effect on *Cdh1* and *Cxadr* promoters was scarce.

In further support to the role of C/EBPβ in maintaining cellular levels of E-cadherin and CAR, we showed that knockdown of C/EBPβ led to decreased mRNA levels of *Cdh1* and *Cxadr*. What is more, we demonstrated that C/EBPβ dissociated from *Cdh1* and *Cxadr* promoters during TGF-β1-induced EMT.

Presented study suggests that C/EBPβ is able to inhibit EMT and restore the TGF-β response towards growth inhibition. Nevertheless, as C/EBPβ failed to fully revert EMT, further studies are required to show the role of additional transcription factors in this process. Furthermore, C/EBPβ was shown to transcriptionally activate genes encoding the epithelial junctions proteins E-cadherin and CAR. Finally, the results identify miR-155-mediated loss of C/EBPβ as a mechanism, promoting breast cancer progression by switching the TGF-β response from growth inhibition to EMT, invasion and metastasis.

3.3 STUDY III

CCAAT-enhancer binding protein β (C/EBPβ) is a transcription factor playing a critical role in mammary gland development and breast cancer progression. Our previous study indicated that loss of C/EBPβ increases metastatic spreading of mouse mammary tumor cells, but did not elucidate the mechanism by which C/EBPβ expression affects metastasis. Therefore, this study aimed at explaining C/EBPβ link with metastasis formation and determining the relationship between C/EBPβ and survival of breast cancer patients

In order to test the hypothesis that C/EBPβ is associated with progression of breast cancer, a tissue microarray containing 137 breast cancer patient samples was immunohistochemically stained for C/EBPβ. Subsequently, a qualitative scoring of C/EBPβ staining was performed in relationship to progression of breast cancer. This descriptive analysis showed that strong nuclear positive staining was present in the normal breast tissue as well as in some ductal cancer *in situ* (DCIS). However, C/EBPβ expression in DCIS was lower compared to expression in normal breast tissue and in many nodules was mostly present in their basal layer. These nodules were surrounded by desmoplastic stroma infiltrated with lymphocytes. Thus, C/EBPβ staining intensity was lower in case of inflammation combined with desmoplastic reaction in the tumor stroma. This phenomenon was strongly visible particularly in areas of microinvasive cancer. The decreased C/EBPβ expression was observed in areas of invasive cancer, especially low in areas with strong lymphocytic infiltration. This observation indicates that C/EBPβ might play role in progression of breast cancer and appearance of inflammation.

All samples were analyzed according to 0-2 scale by a pathologist and related to information concerning patient survival. Cox proportional hazards models and Kaplan-Meier analysis were used to assess the effect of C/EBPβ expression on overall survival (OS) in univariate and multivariate analysis, adjusted for established prognostic factors. All parameters were assessed in univariate models using Cox proportional hazards models and factors significant at $p<0.05$ were included in the final model. In univariate analysis, independent predictors of overall survival (OS) were C/EBPβ expression and tumor size. Lack of C/EBPβ expression in patient samples was associated with shorter overall survival of breast cancer patients as compared with higher C/EBPβ expression. These results indicate that C/EBPβ expression is significantly associated with overall survival, but also related to relapse-free survival (RFS).

In order to study the effect of C/EBPβ on breast cancer, a mouse 4T1 model of breast cancer implantation was employed. Two derivatives of 4T1 cells were used – one referred to as sh C/EBPβ, expressing wild type C/EBPβ and second one described as sh C/EBPβ, with silenced expression of C/EBPβ. Both tumor cell lines were subcutaneously implanted into two groups of syngeneic BALB/c mice and subsequently, tumor growth, CTC dissemination and metastasis formation was compared between the groups.

Loss of C/EBPβ expression affected tumor growth, as well as morphology. C/EBPβ-silenced tumors grew smaller as compared with non-silenced tumors, even though there was no difference in proliferation rate assessed by *in vitro* assay. This result suggests that tumor growth is not directly controlled by C/EBPβ, but rather affected by *in vivo* factors. Additionally, a striking morphological difference was observed between the tumors. While C/EBPβ-expressing tumors were characterized by large central necrosis, C/EBPβ-silenced tumors formed solid tumors with extensive, pushing border growing pattern and inflammation around them.

To further characterize the effect of C/EBPβ on the tumor, tumor vasculature was firstly analyzed by immunohistochemical staining for endothelial cell marker CD31. We observed more vessels in C/EBPβ-silenced tumors as compared with non-silenced tumors.

The study of the tumor vasculature was followed by whole mount immunohistochemistry on tumors, which were stained for both CD31 and a pericyte marker NG2. Subsequently, results were analyzed by Visiopharm software. Despite previous results concerning differences in

vessel number, no significant differences in vessel morphology were found, when assessing features such as vessel length, area, pericyte coverage of the vessels and number of branchpoints, which were similar in both groups.

To study the effect of C/EBPβ on tumor spreading and formation of metastasis, focus was put on the presence of CTCs in blood and bone marrow and appearance of metastatic nodules in the distant organs. In this model, the cells were labeled with EGFP, what allowed for calculating the number of CTC in blood and bone marrow. No statistically significant differences in number of CTC in blood and bone marrow between sh control and sh C/EBPβ tumors were observed at the endpoint of the experiment (2 months following primary tumor excision).

Next, morphological analysis of the lungs of tumor-bearing mice was performed to study if C/EBPβ knockdown affected metastatic spread. The ratio of mice with lung metastasis/without metastasis was significantly higher in mice carrying C/EBPβ knockdown versus control 4T1 tumors, what indicated that loss of C/EBPβ promotes metastatic spread of mammary 4T1 tumors. Moreover, morphological analysis showed that lungs of mice carrying C/EBPβ knockdown had more prominent chronic inflammation versus lungs of C/EBPβ-expressing mice. This observation is in agreement with the fact that C/EBPβ affects formation of inflammatory process, as was already found in human TMA analysis.

To unravel the mechanism of how C/EBPβ affects formation of metastasis, a microarray analysis was performed by using RNA extracts from 4T1 cells expressing C/EBPβ shRNA or control shRNA. Gene profiling revealed a set of 559 genes that were statistically different between sh control and sh C/EBPβ cells. Analysis done by Panther DB service indicates that inflammatory group of genes was the most significant among all genes. Thus, as shown on Fig. 4A, main focus was put to analysis of these genes. Representative genes included MHCIIα, MHCIIβ and HLACIIγ. Additionally, chemokines were also affected, such as CCL5, CCL7 and CCL8.

To follow the chemokines analysis, the cells were further analyzed by chemokine protein array, which did not confirm previous finding. However, many other chemokines were upregulated in the sh C/EBPβ cells as compared with sh control cells, such as CCL2, CCL6, CCL12, CCL27, CCL28, chemerin, CXCL16 and IL-16.

To find if a similar pattern of differences could be observed in the tumor, the tumors dissected from mice were also profiled by microarray analysis, using RNA extracts from 4T1 tumors expressing C/EBPβ shRNA, or control tumors shRNA. In this setup only 135 genes were upregulated more than 1.3 fold. The results were further processed with Panther DB service and in this case, immunological group of genes was also the most representative. Many of the genes on this list are still uncharacterized and have unknown function, however it is striking that CD3+ leukocytes were first on the list with difference equal 5 times fold. These results clearly show that C/EBPβ is involved in modulation of immunological response in the tumors.

Taken altogether, the results of human breast cancer study, morphological analysis of mouse tissue and tumor growth supported the microarray data and suggested that C/EBPβ is involved in metastatic process by controlling the expression of a set of genes involved in inflammatory formation. To confirm this finding, sh C/EBPβ tumors and sh control tumors were stained for

CD45, CD3, CD4 and MHCII. The analysis showed that the inhibition of C/EBPβ leads to an increased expression of MHCII followed by accumulation of CD45, CD3 and CD4-positive lymphocytes in the tumors. This was the most striking and significant difference between the tumors as shown by morphological, microarray data and immunohistochemical analysis.

To study, if the effect of C/EBPβ on tumor growth and metastasis can be reversed by specific treatment, BALB/c mice bearing sh C/EBPβ tumors were treated with anti-CD3 or anti-CD4 antibodies. The results have shown that sh C/EBPβ tumors grew much bigger when treated with either CD3 or CD4 antibodies as compared with non-treated sh C/EBPβ tumors, even though normally C/EBPβ knockdown decreases tumor growth. Moreover, the treatment led to diminishing the differences in tumor morphology. Although no effect of treatment on the number of CTCs in the blood or bone marrow was observed in this model, the experiment confirmed involvement of CD4 T-cells in C/EBPβ-dependent tumor growth. Similar conclusion was drawn from a study in T-cell deprived nude mice, which have no CD3 and CD4 cells at time of experiment, where C/EBPβ-silenced tumors grew bigger than non-silenced tumors. What is more, both groups had similar ratio of lung metastasis, what shows that in this model, C/EBPβ effect on metastasis formation was attenuated as compared with experiment on immunocompetent mice.

To summarize the results of presented study, C/EBPβ is a predictor of overall survival in breast cancer patients, and affects tumor growth, morphology and lung metastasis formation in murine 4T1 breast cancer model. The mechanism of metastasis formation is associated with immunological response, which depends on C/EBPβ-mediated activation of MHCII and CD4+ lymphocytes.

3.4 STUDY IV

Colorectal cancer is one of the leading causes of cancer-related deaths worldwide. Since in most of the cases liver metastasis is responsible for the patient mortality, a significant effort needs to be done to recognize factors controlling metastatic process, what would contribute to improving future patient therapies. Presented study identified CCL7 chemokine as one of such factors and aimed at studying CCL7 role in colon cancer progression *in vivo*. Moreover, human tissue microarray (TMA) was used to determine associations between expression of CCL7 receptors and clinical data from colorectal cancer patients.

In our study, we firstly analyzed crosstalk between murine MSCs and colon colon carcinoma CT26 cells *in vitro*. We showed that CT26 cells have higher proliferation rate, when cocultured in direct contact with MSCs after 24, 48 and 72h. Interestingly, CT26 proliferation rate was not affected when cells were cocultured with MSCs in transwell setting or subject to MSC-conditioned media. Additionally, we demonstrated increased migratory properties of CT26 cells stimulated with MSCs as chemoattractant.

Subsequently, DNA microarray was employed to find the differences in chemokines and their receptors expression between MSCs and CT26 cells. We showed that these cells differ in terms of chemokines and chemokine receptor expression patterns. We observed that CCL7 expression was upregulated, while CCR1 expression, which is CCL7 receptor, was downregulated in MSCs vs. CT26 cells. Moreover, similar results concerning CCL7 and CCR1 were obtained by PCR array analysis using cocultured CT26+MSCs compared to

CT26 cells. Next, ELISA was done to confirm obtained results on protein level. Analysis revealed that MSCs cocultured with CT26 cells in transwell system had significantly higher concentration of CCL7 compared with MSCs in monoculture. Additionally, ELISA showed that CT26+MSC coculture secreted more CCL7 compared to CT26.

To check if recombinant CCL7 affects proliferation rate of CT26 cells *in vitro*, we performed MTT proliferation assay. However, none of studied concentrations of CCL7 led to a change in proliferation rate of CT26 cells. Afterwards, with use of lentiviral transduction, we created a CT26-derived cell line overexpressing CCL7, called mCCL7+, and a control cell line, called blank control. CCL7 overexpression in mCCL7+ cells vs blank control cells was confirmed by ELISA. Comparison of produced cell lines showed no difference in proliferation rates, what once more confirmed that CCL7 has no effect on proliferation of CT26 cells *in vitro*. Surprisingly, scratch assay did not show difference in migration between compared cell lines.

In order to assess effect of CCL7 overexpression on tumor development *in vivo*, blank control or mCCL7+ cells were injected subcutaneously into mice. Study revealed that mCCL7+ cell implantation led to acceleration in the early phase of tumor growth (between day 5 to day 11), when mCCL7+ tumor-bearing mice had larger tumors compared with blank control-injected mice. Nevertheless, already 11 days post-implantation tumors continued to grow in a similar pace in both groups.

Morphological analysis of the isolated tumors showed no striking differences between the two tumor types. However, analysis of murine lungs isolated two months after tumor excision revealed that mCCL7+ cell line injection resulted in increased lung metastasis by 33% in comparison with control mice. This observation indicated that overexpression of CCL7 stimulates metastatic dissemination of CT26 tumors to the lungs. In addition, lungs from CCL7-overexpressing tumor-bearing mice had extensive intra-alveolar bleedings, absent in blank control-implanted mice.

Next, real time PCR was performed in order to determine and compare expression levels of CCL7 receptors (CCR1, CCR2 and CCR3) in blank control, as well as mCCL7+ cell line. We found that the level of CCR1 mRNA was lower by almost 30% in mCCL7+ cells compared with blank control cells. On the other hand, the level of CCR2 mRNA was almost two times higher in mCCL7+ cells compared with blank control cells. Additionally, no CCR3 mRNA was detected, what suggested that the analyzed cell types express only CCR1 and CCR2.

Finally, in order to assess the relationship between CCL7 receptor expression and clinical patient data, a tissue microarray (TMA) containing colorectal cancer patient samples was immunohistochemically stained for CCL7 receptors (CCR1, CCR2 and CCR3). Analysis performed by pathologist revealed different grades of expression of CCL7 receptors, assessed as low or high. Descriptive data analysis showed that 73% of patients who developed lymph node metastases, had high expression of CCR2, compared with only 27% of metastasis-positive patients having lower CCR2 expression. Moreover, high CCR2 expression in analyzed patient samples was associated with shorter relapse-free survival (RFS) of colorectal cancer patients compared with low expression of CCR2. In addition, high CCR2 expression was associated with shorter overall survival (OS) of colorectal cancer patients compared with low CCR2 expression, what was subsequently confirmed by Kaplan-Meier analysis. To sum up, obtained results indicate that high CCR2 expression is significantly associated with OS of colorectal cancer patients. In contrast, no associations of CCR1 or CCR3 with patient survival were found.

Altogether, presented study indicates that overexpression of CCL7 affects early phase of tumor growth and lung metastasis in mouse CT26 colon cancer model and CCR2, as a CCL7 receptor, predicts overall survival of colorectal cancer patients. However, additional work needs to be done in order to discover the mechanism, through which CCL7 regulates metastasis formation and to confirm the role of CCR2-CCL7 axis in this process.

4 CONCLUSIONS

Mesenchymal stem cells and their role in blood vessel formation have been widely discussed in vascular biology field. In **study I**, we identified MSCs derived from adventitia as a source of cells which contribute to intimal hyperplasia. Specifically, CD90+, CD44+ and sca-1+ cells were dominant subpopulations of MSCs that migrated towards the intima. Moreover, these cells expressed CCR2, a receptor for MCP-1, which was the most potent chemokine controlling migration of analyzed MSCs. This study not only confirmed the presence of MSCs in the blood vessels, but also explained their role in intimal hyperplasia, what contributes to better understanding of this process.

Apart from their function in vascular formation and remodeling, MSCs have an important role in tumor progression. However, their impact on this process still remains controversial. In **study IV**, we analyzed the crosstalk between MSCs and colon cancer. *In vitro* studies showed that MSCs increase proliferation rate, as well as migration of mouse CT26 colon carcinoma cells. Moreover, multiple crosstalk analyses revealed MSCs have higher expression of CCL7 chemokine, compared with CT26 cells. Also, cocultured CT26+MSC secrete more CCL7, when compared with CT26 or MSCs in monoculture. Subsequent *in vivo* experiments showed that overexpression of CCL7 quickens the early phase of tumor growth and leads to higher lung metastasis rate in tumor-bearing mice compared to control mice. This observation indicates that CCL7 affects lung metastasis. However, the mechanism behind that relationship remains to be elucidated. Additionally, we identified CCR2 as CCL7 receptor that predicts overall survival of colorectal cancer patients. Namely, higher expression of CCR2 was associated with shorter OS. Further studies need to be performed in order to confirm the role of CCR2-CCL7 axis in metastasis formation and its link to survival of the patients.

Tumor progression is a complicated process regulated by several various factors, such as C/EBPβ transcription factor. In **study II** we analyzed the relationship between EMT and C/EBPβ expression in breast cancer. Human samples analysis demonstrated that lower C/EBPβ expression was associated with triple-negative tumors and mouse experiment confirmed that loss of C/EBPβ was observed in E-cadherin-negative areas of the tumors. We showed that miR-155-mediated loss of C/EBPβ was specifically associated with EMT. Moreover, silenced expression of C/EBPβ in breast cancer cells led to higher invasiveness and lung metastasis in tumor-bearing mice compared to control mice. In addition, C/EBPβ was demonstrated to be transcriptional activator o genes encoding epithelial junctions proteins (E-cadherin and CAR). Described study suggests that C/EBPβ can inhibit EMT and restore response of TGF-β towards growth inhibition. Nonetheless, deeper studies are needed to show the function of additional factors in this process, as C/EBPβ was not able to fully revert EMT on its own.

Study III focused on the role of C/EBPβ in metastasis formation and its effect on tumor morphology. Firstly, we identified C/EBPβ as a predictor of OS of breast cancer patients. Secondly, *in vivo* analysis showed that loss of C/EBPβ affects tumor growth and morphology

in mouse 4T1 breast cancer model. Namely, C/EBPβ-silenced cells caused formation of smaller in size, yet intact and well vascularized tumors, compared to bigger, but necrosis-containing control tumors. Furthermore, C/EBPβ knockdown promoted lung metastasis formation by triggering inflammation. Specifically, inflammatory response was triggered through MHCII activation and accumulation of CD4+ lymphocytes in the sh C/EBPβ tumors. Finally, blocking CD4-mediated inflammation in 4T1 mice attenuated the effect of C/EBPβ on tumor growth, morphology and metastasis formation. Presented study not only found a new predictive factor for OS of breast cancer patients, but also provided new insights on effect of C/EBPβ on breast cancer progression.

Both regenerative medicine and cancer field need new therapy targets, as well as predictive factors. This work not only contributed to broadening the knowledge about role of mesenchymal stem cells in vascular structure and remodeling, but also shed new light on C/EBPβ as a factor affecting metastasis, tumor growth and morphology. Moreover, C/EBPβ was identified as a predictor of overall survival in breast cancer patients, while CCL7, in colorectal cancer patients, what has high importance for clinical oncology.

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