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Identification of disease-promoting stromal components by comparative proteomic and transcriptomic profiling of canine mammary tumors using laser-capture microdissected FFPE tissue

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1. Zusammenfassung

Das Krebs-assoziierte Stroma (CAS) beeinflusst die Entwicklung von Mammakarzinomen (mCA) in hohem Masse. Canine einfache mCA sind relevante Modelle für humane mCA, auch in Hinsicht auf CAS. Während Veränderungen des CAS-Transkriptoms in mCA gut beschrieben sind, ist unklar, inwieweit sich dessen Proteom verändert. Das Ziel dieser Arbeit war es, zu untersuchen, wie sich das Proteom im CAS caniner mCA von normalem Stroma unterscheidet, und inwiefern dies mit dem Transkriptom korreliert. Dafür analysierten wir CAS und normales Stroma mittels Laser-Mikrodissektion (LCM) und LC-MS/MS in einer Kohorte von 14 formalin-fixierten, Paraffin-eingebetteten (FFPE) caninen mCA, die zuvor mittels LCM-RNAseq charakterisiert worden waren. Die Resultate zeigten deutliche Unterschiede der Proteinzusammensetzung zwischen normalem Stroma und CAS, welche vor allem durch Veränderungen in der extrazellulären Matrix, dem Zytoskelett und Zytokinen gekennzeichnet waren. Proteomik und RNAseq zeigten ein erhebliches Mass an Korrelation, insbesondere für stark deregulierte Zielstrukturen und aktivierte Signalwege. Schliesslich validierten wir die Hochregulierung von LTBP2, IGFBP2, COL6A5, POSTN, FN1, COL4A1, COL12A1, PLOD2, COL4A2 und IGFBP7 in CAS auf Proteinebene und zeigten ihren negativen prognostischen Wert für humanen Brustkrebs auf. In Anbetracht der Relevanz des caninen mCA als Modell für die menschliche Erkrankung haben diese Resultate Implikationen für Brustkrebs beider Spezies.

Schlüsselwörter: Tumor-Mikroumgebung, vergleichende Onkologie, Brustkrebs, Tumorstroma, canine Mammakarzinome

2. Summary

Cancer-associated stroma (CAS) profoundly influences progression of tumors including mammary carcinoma (mCA). Canine simple mCA represent relevant models of human mCA, notably also with respect to CAS. While transcriptomic changes in CAS of mCA are well described, the extent to which its proteome changes remains unclear. Therefore, we sought to gain insight into the proteomic changes in CAS and compare them with transcriptomic changes in the same tissue. To this end, we analysed CAS and matched normal stroma using laser-capture microdissection (LCM) and LC-MS/MS in a cohort of 14 formalin-fixed paraffin embedded (FFPE) canine mCAs that we had previously characterized using LCM-RNAseq. Results revealed clear differences in protein composition between CAS and normal stroma, which were characterized by changes in the extracellular matrix, the cytoskeleton, and cytokines. Proteomics and RNAseq showed a substantial degree of correlation, especially for highly deregulated targets and activated signaling pathways. Finally, we validate upregulation of LTBP2, IGFBP2, COL6A5, POSTN, FN1, COL4A1, COL12A1, PLOD2, COL4A2, and IGFBP7 in CAS on the protein level and demonstrate their adverse prognostic value for human breast cancer. Given the relevance of canine mCA as a model for the human disease, these results have implications for breast cancer of both species.

Keywords: Tumor microenvironment, comparative oncology, breast cancer, tumor stroma, canine mammary carcinoma

3. Abbreviations

| | |
|-------------|--|
| bFGF | basic fibroblast growth factor |
| CAF | cancer-associated fibroblasts |
| CAS | cancer-associated stroma |
| COL | collagen |
| ECM | extracellular matrix |
| ER | estrogen receptor |
| FAP | fibroblast activation protein |
| FISH | fluorescence in situ hybridization |
| FC | fold change |
| FDR | false discovery rate |
| FFPE | formalin-fixated, paraffin-embedded |
| HER2 | human epidermal growth factor receptor |
| LCM | laser-capture microdissection |
| mAb | monoclonal antibody |
| mCA | mammary carcinoma |
| MMP | metalloproteinases |
| PCA | principle component analysis |
| PDGFRB | platelet-derived growth factor receptor beta |
| RNAseq | RNA sequencing |
| TAMs | tumor-associated macrophages |
| TGF β | transforming growth factor beta |
| TILs | tumor infiltrating lymphocytes |
| TNF | tumor necrosis factor |
| VEGF | vascular endothelial growth factor |

4. Introduction

4.1. Breast cancer is a worldwide major health concern

Cancer is a term used to describe a variety of diseases that occur when the organism loses control over mitotic propagation of single cells (LYNCH, 1987). This results in uncontrolled division of cells that can affect the whole body and leads to the formation of so-called tumors. Depending on their behavior with respect to healthy tissue, such tumors can be malignant or benign. While benign tumors remain in their original location, malignant tumors grow quickly, can invade the surrounding tissue and thereby spread to other parts of the body, also known as metastasis. Over 100 different types of cancer are known to affect humans. Despite intense research efforts to promote knowledge of cancer biology and therapy, the number of deaths that are attributed to cancer has constantly risen over the past decades and currently presents the second leading cause of death worldwide (World Health Organization, 2020). In 2020, 19.3 million new cases and 10 million death due to cancer were reported (Sung et al., 2021). Of all new cancer cases, 2.261.419 cases (11.7%) and 684.996 of all deaths are due to breast cancer (6.9%). Indeed, breast cancer is the most commonly diagnosed cancer in women both worldwide and in Switzerland. Over a lifetime, 40% of the Swiss population gets diagnosed with cancer and 3 out of 10 people will die because of it (Heusser et al., 2017). In 2020, Switzerland counted 60.483 new cases of cancer of which 12% were breast cancer (International Agency for Research on Cancer, 2021). While the incidence of breast cancer has slowly risen over the past decades in Switzerland, the death rate has declined by 30% thanks to early diagnosis and improved therapy (Heusser et al., 2017). Yet, 2020 had a negative impact on cancer rates and outcomes as COVID-19 led to elevated mortality rates, delayed diagnosis, altered treatment pathways to reduce the risk of exposure to the virus, reduced available therapy options due to suspension of clinical trials (Liang et al., 2020; Richards et al., 2020). Accordingly, by systematically reviewing seven major cancer types and three treatment modalities, Hanna et al. corroborate the hypothesis that a delay in cancer treatment leads to a significant increased mortality rate (Hanna et al., 2020). Moreover, cancer research that relies on cancer patient samples was limited due to the lack of availability, thereby providing negative impact on progress in cancer treatment (Mulholland, 2021). Thus, cancer in general and breast cancer in particular remains a major worldwide health concern that requires continued efforts to further our understanding of the disease to improve its therapy and further increase patient survival.

4.2. The importance of cancer-associated stroma in cancer biology

The molecular basis of cancer is rooted in transformation of healthy cells towards cancer cells through genetic and/or epigenetic changes. Accordingly, the majority of cancer research has focused on understanding the behavior of these cancer cells,

which often derive from an epithelial origin. However, more recent progress has revealed that, much like a flower seed cannot grow without the appropriate soil, cancer cells cannot survive on their own and need appropriate stromal surroundings to sustain their survival and growth. This so-called “seed-and-soil” theory was already proposed back in 1889 by Stephen Paget, an English surgeon (Paget, 1889), but its molecular underpinnings have only begun to be investigated in detail over the last two decades. Through this progress it has become abundantly clear that the microenvironment that surrounds cancer cells, the so-called cancer-associated stroma (CAS), has profound effects on the development and survival of tumor cells, and thereby strongly influences clinical aspects of the disease (Gandellini et al., 2012; Hanahan & Coussens, 2012; Song et al., 2019). Indeed, CAS has been shown to profoundly influence most of the hallmarks of cancer in a wide variety of tumors, including breast cancer (Hanahan & Coussens, 2012). CAS is composed of a large variety of normal, non-malignant cells including fibroblasts, immune cells, vascular cells, adipocytes, and others that reside in an insoluble extracellular matrix (ECM) composed of collagens and other structural elements. Under physiological circumstances, healthy stroma serves as an important barrier to prevent epithelial transformation. However, in the vicinity of malignant tumor cells, stroma is reprogrammed into CAS. This reprogramming of normal stroma to CAS is strongly driven by the adjacent cancer cells that produce a range of cytokines, growth factors and proteases which modify the surrounding stromal environment to their own advantage (Gkretsi et al., 2015; YUAN et al., 2016). In the following, I will shortly touch upon individual components of the CAS and their role on tumor biology.

Cancer-associated fibroblasts

As the main ECM-producing cells, fibroblasts are among the most abundant cells in the stroma. When normal fibroblasts are in close vicinity of cancer cells, this leads to their activation into so-called cancer-associated fibroblasts, or CAFs. CAFs are fibroblasts that differ phenotypically and functionally from their normal counterparts and strongly resemble myofibroblasts, which occur during physiological wound healing (Micallef et al., 2012; Östman & Augsten, 2009). In normal tissue, fibroblasts are key components of wound healing and inflammation and are known to be able to protect against malignant progression of epithelial cells (Coussens & Werb, 2016). During wound healing, fibroblast undergo transformation into myofibroblasts, which have an increased contractility that is important to mediate closure of the wound. Similarly, CAFs strongly resemble myofibroblasts in many aspects including increased contractility and it has been suggested that they can originate from various cell types. Through various cytokines and signaling molecules, neoplastic cells are able to activate fibroblasts into CAFs, which in turn strongly promote tumor progression (Bhowmick et al., 2004; Kalluri, 2016; Marsh et al., 2013; Sahai et al., 2020; Tripathi et al., 2012). CAFs influence the tumor microenvironment by secreting and/or activating cytokines, growth factors, nutrients and proteases, all of which leads to tumor growth, expansion and dissemination of the pre-neoplastic epithelial cell population (Bissell & Radisky, 2001; Franco et al., 2010; Hanahan & Coussens,

2012). The influence of CAFs is evident also from the fact that the presence of specific subsets of CAFs have been shown to correlate with poor survival and chemoresistance in breast and lung cancer (Huelsken & Hanahan, 2018; Su et al., 2018).

Immune cells

Another important component of the CAS is formed by infiltrating immune cells. In a physiologically regulated environment, inflammation is a self-limiting process, meaning that pro-inflammatory cytokines are followed by anti-inflammatory cytokines to keep the inflammatory reaction under control. Any dysregulations of this process can lead to abnormalities, and ultimately, pathogenesis. Normally, the immune system recognizes transformed epithelial cells and removes these and thereby provides a very important defense mechanism to protect the organism from tumor development. Accordingly, an inflammatory component is present in the microenvironment of most neoplastic tissues, as the immune system recognizes illegitimate cell proliferation. However, cancer cells often learn how to avoid, blunt or redirect the immune attack, and sometimes even manage to use this continuous inflammation in their favor. For example, cancer cells can use the immune cells as a source of tumor-supportive cytokines (Colotta et al., 2009; Mantovani et al., 2008). Some of those cytokines can for instance activate transcription factors, such as NF- κ B, STAT3, and AP-1 which in turn induce expression of genes that stimulate cell proliferation and survival (Grivennikov et al., 2010).

One main group of immune cells found in tumors are lymphocytes, which include T-cells, B-cells, and natural killer (NK) cells. Lymphocytes that leave the blood stream and infiltrate the tumor are called tumor infiltrating lymphocytes (TILs). They can be found between tumor cells or in the CAS in most types of solid tumors (Badalamenti et al., 2019). As they have the capability to kill cancer cells, high amounts of TILs are usually correlated with a good prognosis (Bremnes et al., 2016; Mahmoud et al., 2011; Toor et al., 2019; Wouters & Nelson, 2018). Accordingly, Verma et al. demonstrated that stromally located TILs that are positive for CD20 (B cells) or CD4 (T cells) in ER negative breast cancer cases correlate with improved outcomes (Verma et al., 2020). Ali et al. revealed an impact of cytotoxic (CD8+) lymphocytes in ER-negative (both HER2-positive and HER2-negative) and ER-positive/HER2-positive breast cancer on the survival rate (Ali et al., 2014). Women with presence of CD8+ cells show a higher survival rate than women without. In addition, the location of T cells, whether they are found in tumoral or stromal tissue, does not impact on the result (Ali et al., 2014). Furthermore, TILs can be used for immunotherapy by separation, amplification, and blood-transfusion. These TILs are isolated from tumor tissue of patients, so they are not genetically modified. Only the lymphocytes that show a specific killing effect on tumor will be amplified and later used in blood transfusions after the patient has received chemotherapy with the aim of targeting cancer cells that were not eradicated by it (Lin et al., 2020).

Other important tumor-infiltrating cell types are macrophages that are derived from monocytes. By producing various angiogenic and lymphangiogenic growth factors,

different proteases and cytokines, macrophages play an important role in chronic inflammatory processes. They can be divided in two main groups, the classically activated “M1 macrophages” and the alternatively activated “M2 macrophages”. In contrast to M2 macrophages, M1 macrophages react in a pro-inflammatory way, whereas M2 are involved in anti-inflammatory responses and are associated with a worse clinical presentation (Larionova et al., 2020; Pan et al., 2020; Steidl et al., 2010). Cancer cells can influence macrophages to turn into tumor associated macrophages (TAMs) that suppress antitumor immunity (Murray & Wynn, 2011). The number of TAMs and intratumoral lymphatics can also be used as prognostic markers for different types of cancer, for example colorectal cancer, where high numbers correlate with poor survival (Helm et al., 2014). A meta-analysis of sixteen studies related to breast cancer revealed that a high number of TAMs correlate with malignant biological behavior and poor overall survival (Zhao et al., 2017). Another meta-analysis showed similar results for pancreatic cancer (M. Yu et al., 2019).

Angiogenesis

For growth and progression, tumors require a functioning vasculature that provides a constant supply of oxygen and nutrients and enables removal of metabolic waste products. To cater to this demand, tumors induce formation new blood and lymph vessels. Indeed, it has been estimated that, without appropriate blood supply, tumors are not able to become larger than 1–2 mm³, mostly because simple diffusion of nutrients is not efficient enough to sustain the increased demands (Nishida et al., 2006). To induce angiogenesis, tumors express a series of angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenic transforming growth factor (TGF)-alpha, TGF-beta, tumor necrosis factor (TNF), granulocyte colony-stimulation factor, placental growth factor, interleukin-8, hepatocyte growth factor, epidermal growth factor, and many others.

ECM

All previously discussed cells are embedded in the ECM, which is a three-dimensional, non-cellular structure that provides a physical scaffold to ensure proper tissue architecture. Its components are proteoglycans, hyaluronic acid, and fibrous proteins (Egeblad et al., 2010). ECM is present in all tissues and is essential for life. We can differentiate between two main types of ECM, the interstitial connective tissue matrix and the basement membrane. The connective tissue matrix surrounds cells and provides a scaffold for the tissue, whereas the basement membrane can be found as a lining between the epithelium and surrounding stroma. The ECM contributes to paracrine cellular signaling and regulates many cellular processes including growth, migration, differentiation, survival, homeostasis, and morphogenesis (Frantz et al., 2010; Hynes & Naba, 2012; Theocharis et al., 2016). To be able to influence those processes, the ECM interacts with epithelial cells via for example ligands such as integrins or it releases growth factors, such as epidermal

growth factor (EGF), bFGF and others (Miyamoto et al., 1996; Williams et al., 1994). These also allow bidirectional communication between cells and ECM macromolecules. Moreover, The ECM is subject to a constant turnover and change through enzymes such as collagenases and matrix metalloproteinases (MMPS) that adapt the ECM according to structural needs of the tissue (Bonnans et al., 2014). Similarly, during cancer progression, strong changes in the abundance of different types of collagens can be observed in the ECM. Some of these collagen remodeling biomarkers can even be used to measure tumor activity in regard of progression and metastasis (Kehlet et al., 2016).

In summary, a lot of knowledge regarding the role of CAS in tumor biology has been gained over the last two decades. A picture starts to emerge in which bidirectional interaction of tumor cells with the surrounding CAS is an absolute prerequisite for cancer to develop. Similarly, increased understanding of this dialogue has led to the development of several very promising therapeutic approaches, including immunotherapies. However, our understanding regarding CAS reprogramming and the molecular dialogue between CAS and cancer cells remains incomplete, especially so in actual patient samples. More detailed insight into the crosstalk of malignant cells with their surroundings has the potential to unravel novel therapeutic approaches to help treat patients suffering from cancer.

4.3. Canine mammary tumors as a model for human breast cancer

Cancer can affect a big variety of cells and is therefore used as an umbrella term for a large group of diseases. Even the same type of cancer can vary in for example behavior, symptoms, and location between individuals. This is exactly where “comparative oncology” might provide an advantage over historically used classical rodent tumor models. The basic concept of “comparative oncology” is to integrate the study of naturally occurring cancers in animals with that of human cancer and thereby facilitate identification of central pathways that are responsible for the disease. While it might seem quite unusual to use the dog as a model organism for humans at first sight, tumors in dogs share a lot of similarities with human cancer, and also present several advantages over widely used rodent models for cancer research. For instance, the environment that organisms are exposed to is a very important factor that strongly influences cancer development. Dogs often share the same environment as humans and therefore are exposed to much of the same risk factors. A lot of research is done in rodents or other laboratory animals which develop cancer under different conditions compared to humans. For example, tumor progression is extremely fast in mice compared to humans which limits the opportunities to study novel therapeutics. Instead, pets like dogs and cats are found to share more similarities especially in tumor development and progression (Hansen & Khanna, 2004; Vail & Macewen, 2000). In general, physiology of humans is more

comparable to dogs than to rodents regarding anatomy, size, physiology, metabolism, immunology, and genetics. And dogs and humans often show similar clinics and pathophysiology when it comes to cancer (Munson & Moresco, 2007). Furthermore, sequencing of the dog genome has demonstrated the presence of more consensus between the genome of human and dogs than between humans and mice (Hoffman & Birney, 2006; Lindblad-Toh et al., 2005).

Based on clinical, histological, and molecular similarities, canine simple mammary carcinomas (mCA) are considered an excellent model for human breast cancer, also because they overcome several of the limitations of xenograft or genetically modified rodent tumor models (Liu et al., 2014; Queiroga et al., 2011; Schiffman & Breen, 2015). Indeed, canine simple mCA not only emulate the biology of human mCA but also feature many of the genomic aberrations found therein (Liu et al., 2014; Queiroga et al., 2011b). mCA are the most frequent tumors in both women and intact female dogs (Salas et al., 2015). In contrast, female dogs that get neutered before their first estrus cycle, show an incidence for mCA of 0.05% compared to intact female dogs, which is thought to reflect the hormonal influence during tumor development (Rivera et al., 2009; Zatloukal et al., 2005). Similarly, women with a BRCA1 germline mutation can reduce their breast cancer risk by undergoing a prophylactic bilateral oophorectomy before menopause (Rebbeck et al., 1999). Canine simple mCA are malignant epithelial neoplasms that infiltrate the surrounding tissue, whereby they induce a strong stromal response, and can also give rise to metastases (Goldschmidt et al., 2011). Importantly, the similarities between human and canine mCA are not only limited to the tumor cells, but also extend to reprogramming of CAS. By analyzing CAS and normal stroma from formalin-fixed paraffin embedded (FFPE) breast cancer tissue using laser-capture-microdissection (LCM) through RNA sequencing (RNAseq) and quantitative PCR (RT-qPCR), our group has recently demonstrated the existence of strong molecular homologies in stromal reprogramming between human and canine mCA (Amini et al., 2017, 2019, 2020; Markkanen, 2019). Hence, comparative study of CAS between human and canine mCA has the potential to reveal interesting new findings with regards to tumor-stroma crosstalk.

While transcriptomic changes in CAS of both human and canine mCA are beginning to be understood, it remains unclear to what extent these differences in mRNA abundance actually translates to the protein level. Thus far, analytic approaches of CAS reprogramming in both human and canine patient samples of mCA have been mostly focused on analysis of RNA, therefore reflecting the transcriptional state of the tissue (Amini et al., 2019, 2020; Finak et al., 2008; Pepin et al., 2012). However, because RNA levels and protein abundance do not necessarily correlate (Greenbaum et al., 2003; Maier et al., 2009; Popovic et al., 2018), it remains entirely unclear to what extent the observed transcriptional changes translate to the protein level in both human and canine mCA. To the best of our knowledge, there is only one report describing proteomic changes in CAS in fresh-frozen samples of human mCA

(Braakman et al., 2017), and none for canine mCA. This striking shortage of data on a highly relevant aspect of tumor biology warrants further investigation.

4.4. The power of prognostic markers

For a cancer patient, the diagnosis is only the first step in a long journey towards recovery. After that, tumor stage and grade must be evaluated to be able to establish an individual therapy and predict survival chances. With new technologies and advances in the field of genomics, the interest in tumor markers that either predict patient's overall outcome, called prognostic marker, or predictive markers, which indicates sensitivity or resistance to a specific type of therapy, have risen strongly over the last decades. Markers can also show both qualities, prognostic and predictive. They are used to detect if certain molecular structures such as proteins are up- or downregulated that are related to survival and prognosis. The ability to discover prognostic markers has increased with easier and more affordable DNA sequencing over the past 4 decades (Shendure et al., 2017). However, it remains a difficult task to find a prognostic marker that is specific for a malignant tumor, able to accurately predict the outcome of most patients, and easy to detect. Finding such markers involves multiple disciplines that need to work closely together in prognostic research. Two very common and widely used markers since decades are tumor size and histological grade (Carter et al., 1989; Donegan, 1997). For breast cancer, lymph node status and hormone receptor status are additional important prognostic factors. The hormone receptor status is routinely assessed, and tumors are categorized in 'hormone-receptor-positive', 'hormone-receptor negative', 'triple-negative' and 'triple-positive'. Tumors that are positive for one or more hormonal receptors can be treated easier than those tumors that do not express either receptor or growth factor. Tumors are tested for the estrogen (ER)- and progesterone (PR)-receptors as well as the human epidermal growth-factor (HER) status. Dunnwald et al. analyzed data of 155.175 women with breast cancer to evaluate a correlation between hormone receptor status and mortality. Women with ER+/PR+ had a lower risk of mortality compared to women with ER-/PR+, ER+/PR- or ER-/PR- (Dunnwald et al., 2007). Furthermore, Slamon et al. evaluated the efficacy of a monoclonal antibody against HER2 in combination with chemotherapy which prolongs survival in patients with metastatic breast cancer compared to patients that only received chemotherapy (Slamon et al., 2001). This emphasizes the prognostic power of the hormone receptor status. The downside of the listed factors is that they are not suitable markers for early diagnosed breast cancer patients (M. J. Duffy et al., 2016, 2017). Molecular prognostic markers can be downregulated, as for example the JAK1 mRNA levels in breast cancer, which also correlates with poor survival (B. Chen et al., 2019). JAK1 is essential for certain cytokines and important for transducing interferon signals. Markers can also be upregulated, like Ki67, which is widely used to measure and monitor proliferation of mammary tumors (Shoker et al., 2001;

Urruticoechea et al., 2005). In general, biomarkers can be analyzed by different methods. The two most commonly used ones are fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) (Kaliyappan et al., 2012; Moter & Göbel, 2000).

To decide whether therapy before or after surgery would be beneficial, it is helpful to use genomic tests to analyze the activity of certain genes in tumors. Growth and spread are tumor characteristics that are influenced by certain genes. With the help of molecular tests like MammaPrint or OncotypeDX, which are commercially available, doctors can predict to a certain degree which pre- or post-operative treatments are necessary (Soliman et al., 2020). Without prognostic markers, a lot of patient would undergo treatments like chemotherapy without any positive impact on the disease while wasting valuable lifetime of a patient. Hence, good prognostic and predictive markers also have the potential to strongly improve patient welfare.

4.5. Aim of the thesis

Given the pivotal impact of CAS on tumor biology, there is ample interest in better understanding the changes that occur in CAS in patient samples. While knowledge regarding transcriptional reprogramming of CAS in both human and canine mCA is starting to emerge, it remains entirely unclear to what extent the observed transcriptional changes translate to the protein level. To the best of our knowledge, there is only one report describing proteomic changes during stromal reprogramming in fresh-frozen samples of human mCA (Gandellini et al., 2012; Hanahan & Coussens, 2012; Song et al., 2019), and none for canine mCA. This striking shortage of data on a highly relevant aspect of tumor biology warrants further investigation. FFPE tissue represents a huge resource of patient material that is routinely prepared in pathology departments and can be easily stored for decades. However, FFPE negatively impacts on the quality and quantity of macromolecules that can be isolated from these tissues, making analysis of RNA or protein from such tissue challenging. Having already established RNA analysis of microdissected areas from such tissue, we set out to define proteomic changes during stromal reprogramming in archival FFPE patient samples and to compare them with transcriptomic changes in the same tissue. To this end, we isolated CAS and matched normal stroma using LCM from a cohort of 14 archival FFPE canine mCAs that we had previously characterized using LCM-RNAseq (Hedegaard et al., 2014; Sinicropi et al., 2012; Tanca et al., 2012) and analyzed them by LC-MS/MS. By doing so, we aimed to address the following key question: i) what are the proteomic changes in CAS from canine mCA, ii) how do the changes on the protein level relate to transcriptional changes observed in these tissues, and iii) can any of the observed changes be used as a prognostic marker for mCA?

Answering of these key questions is expected to significantly improve our knowledge regarding the biology of canine and human CAS and has the potential to unveil novel therapeutic strategies to benefit both canine and human breast cancer patients.

5. Identification of disease-promoting stromal components by comparative proteomic and transcriptomic profiling of canine mammary tumors using laser-capture microdissected FFPE tissue

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This is the first report to comparatively analyze CAS reprogramming in canine mammary carcinomas using proteomic and transcriptomic analysis of FFPE tissue.

My contribution to this paper:

As the first author, I was responsible for isolation of CAS and normal stroma by LCM from all patient cases for proteomic analysis, for data analysis and for writing the first draft of the manuscript.

6. Discussion

Cancer remains one of the most feared diseases as the number of new cases has risen over the past decades to 19.3 million with 10 million deaths in 2020 (Sung et al., 2021). This makes cancer the number two cause of death worldwide. Among women, breast cancer is the most frequently occurring cancer. Despite all the effort that is put into cancer research, there is much we do not understand about cancer, which translates to our inability to cure all cancer patients. While much research has focused on cancer cells, more recent progress has unveiled the pivotal impact of the CAS on tumor growth and progression. It has become clear that cancer cells can reprogram their surrounding stroma and transform it into CAS, which in turn displays tumor-supportive features. Hence, better understanding CAS and its interaction with tumor cells has the potential to unveil novel approaches to improve cancer therapy. Cancer research strongly relies on model organisms. In this respect, comparative analysis of cancer in dogs has tremendous potential to improve our knowledge of the human condition. Particularly canine simple mCA are increasingly viewed as a good model to understand human breast cancer in a comparative setting.

Given the prominent role of CAS in the biology of human breast cancer, it is very likely to be a central actor in canine mCA, too. While CAS reprogramming in canine and human mCA has been analyzed on the transcriptional level, it remains unclear to what extent transcriptomic changes therein translate to the protein level, and what the proteomic landscape of stromal reprogramming looks like in cancer patient samples. Indeed, in many aspects, proteins are the real 'work-horses' of a cell and strongly determine the function of cells, tissues and entire organs. Protein production is influenced by many post-transcriptional mechanisms, which is why mRNA levels do not necessarily correlate with protein abundance (Coppin et al., 2018; Hershey et al., 2012). Therefore, to better understand CAS reprogramming in both canine and human mCA, it is important to know what changes occur in CAS on the protein level. To address this question, we set out to characterize the proteomic changes between normal stroma and CAS in canine mCAs. Our main focus was to gain insight into the following aspects: i) what are the proteomic changes in CAS from canine mCA, ii) how do the changes on the protein level correlate with transcriptional changes in these tissues, and iii) can any of the observed changes be used as a prognostic marker for human mCA?

6.1. Proteomic analysis of CAS and normal stroma from LCM-FFPE patient tissue

To address these questions, we harnessed the validity of spontaneous canine simple mCA as a relevant model of human mCA, that extends also to CAS reprogramming, to gain insight into the proteomic landscape of stromal reprogramming in both human

and canine breast cancer. By investigating proteomic changes in microdissected FFPE patient tissues using LC-MS/MS, we obtained the first dataset to assess proteomic changes between CAS and normal stroma of canine mCA. Our results demonstrate the feasibility to identify proteins from microdissected FFPE patient samples and reveal marked changes in protein composition between CAS and normal stroma in canine mCA. These results contribute to a better understanding of the involvement of stromal genes in development and progression of both canine and human mCA, serve as a basis for further in-depth mechanistic studies of involved genes, and have the potential to reveal both novel prognostic markers and therapeutic targets.

Archival FFPE patient samples represent a huge resource of patient material that is routinely prepared in pathology departments and can be easily stored for decades. However, FFPE negatively impacts on the quality and quantity of macromolecules that can be isolated from these tissues, making analysis of RNA or protein from such tissue challenging. Despite these difficulties, recent years have brought a big improvement in extraction and analysis methods, rendering the analysis of RNA or proteins from FFPE tissue possible while also demonstrating a good consistency between results from FFPE and fresh-frozen tissue (Heaton & Master, 2011; Smith et al., 2013). Bulk analysis of fresh-frozen human breast cancer has revealed significant differences between malignant and matched non-tumor tissue (Drev et al., 2017; Puré & Blomberg, 2018). The positive aspects in terms of quality of extracted macromolecules when using fresh-frozen tissue are heavily counterbalanced by the disadvantages that arise through the need of a high grade of coordination to achieve proper collection and storage. Furthermore, tissue morphology of fresh-frozen tissue is inferior to that of FFPE. And finally, using fresh-frozen tissue precludes the use of most archival samples, which are generally processed as FFPE. Indeed, proteomic analysis has also been shown to work for microdissected FFPE tissue (reviewed in Nissen et al., 2019). Hence, this possibility to analyze specific areas of archival patient samples by both RNAseq and proteomics unlocks a novel dimension of hard-to-analyze samples for investigation.

6.2. The proteomic landscape of CAS and normal stroma in canine mCA

Our proteomic approach revealed several interesting insights into CAS-reprogramming. Importantly, the most significantly deregulated proteins are all produced by stromal cells, thus validating our approach to isolate and analyze stroma. Some of the proteins, such as Collagen type VIII, Fibrillin, LTBP2, are known to be of mainly stromal origin and are upregulated in colorectal cancer (Drev et al., 2017; Puré & Blomberg, 2018) Especially the composition of collagens seemed to shift quite strongly between normal stroma and CAS. The most significantly upregulated collagens that we found in CAS were the following: COL4A1, COL6A3,

COL8A2, COL11A1, COL11A2, COL12A1, and COL15A1. Most of the collagens are produced by stromal cells, and fibrillar collagens and collagen VI for instance are collagens known to be produced by CAFs (reviewed in Nissen et al., 2019). Collagens have been found to have important roles in different types of cancer, for example collagen XII in gastric cancer and ovarian cancer (Duan et al., 2018; Januchowski et al., 2016; Jiang et al., 2019) where it has been associated with drug resistance and poor overall survival, or collagen XI which is also expressed in CAFs (Freire et al., 2014). Overexpression of collagen XI can be found in different types of cancer, for example breast cancer (Freire et al., 2014; Halsted et al., 2008), non-small cell lung cancer (Shen et al., 2016), colorectal cancer (Fischer, 2001) and ovarian cancer (Y.-H. Wu et al., 2014). Its association with invasiveness and malignancy (Freire et al., 2014), poor clinical outcome and capabilities to promote metastasis reflects the importance of collagen type XI in cancer progression (Freire et al., 2014; Jia et al., 2016; Shen et al., 2016). Additionally, genes of collagen XI and XII are also among the most upregulated genes in CAS from human breast cancer (Ma et al., 2009). Hence, the upregulation of these collagens on the protein level is in strong accordance with the findings of other studies and further highlights them as interesting targets for further mechanistic investigation.

Gene ontology analysis revealed a strong involvement of the production and regulation of the TNF superfamily, with the regulating molecules THBS1, LTF, ACP5, and BPI that were all strongly upregulated in CAS compared to normal stroma. TNF is known to be frequently upregulated in human epithelial malignancies such as breast cancer (Boldrini et al., 2000; Katerinaki et al., 2003; Miles et al., 1994). Both tumor cells and tumor-associated macrophages are able to produce TNF. On the cellular level, TNF exerts its effects through its receptors that activate distinct signaling pathways. On one hand, TNF is involved in all stages of human breast cancer development, affecting tumor cell proliferation and survival, epithelial-to-mesenchymal-transformation, metastasis and recurrence (reviewed in Cruceriu et al., 2019). On the other hand, TNF also possesses some anticancer properties through inducing cancer cell death (reviewed in Wang & Lin, 2008). THBS1 (Thrombospondin-1) is a protein that acts in the tumor microenvironment to inhibit angiogenesis, regulate antitumor immunity, stimulate tumor cell migration, and regulate the activities of extracellular proteases and growth factors (Roberts, 2005; Stenina-Adognravi et al., 2018). LTF (Lactotransferrin) is an iron-binding glycoprotein which is stored in specific granules in neutrophils. High amounts of LTF can be found especially in milk and fluids of the digestive tract. It regulates the immune response and protects against infection and septic shock as an antibacterial and anti-inflammatory agent (Conneely, 2001; Ward et al., 2005). Because of its anti-inflammatory function, LTF can inhibit the secretion of TNF (Choe & Lee, 2000; Legrand et al., 2005; Machnicki et al., 1993). Nevertheless, its function in breast cancer is still unclear. ACP5 (Tartrate-resistant acid phosphatase 5), which is essential for bone resorption and osteoclast differentiation, promotes cell motility through the modulation of focal adhesion kinase phosphorylation. Its expression is

found in cells of the mononuclear phagocyte system, in a variety of differentiated cells of the myeloid lineage such as granulocytes (eosinophils), and in osteoclasts (Lamp & Drexler, 2000). Moreover, Gao et al. found ACP5 to be upregulated in lung adenocarcinoma tissues and that high expression of ACP correlated with poor overall survival (Gao et al., 2018). Bactericidal permeability increasing protein (BPI) is an antibacterial and endotoxin-neutralizing protein expressed by epithelial cells. It inhibits the release of TNF, especially when it is LPS-induced (Aloisi et al., 2000). Further analysis of the involvement of the TNF pathway in CAS of canine and human mCA will likely yield interesting data.

The strong role of TGF-beta related signaling that emerged from pathway analysis is well supported by literature. TGF-beta naturally possesses both tumor-suppressive as well as tumor-promoting qualities, depending on the context. As tumor-promoter TGF-beta can support the promotion of tumor growth and invasion, evasion of immune surveillance, and metastasis (reviewed in Korpál & Kang, 2010; and Massagué, 2008). Also associated with the TGF-beta pathway is PAI-1 (plasminogen activator inhibitor-1), a serine protease inhibitor that we found to be strongly upregulated in CAS. PAI-1 inhibits tissue plasminogen activator and urokinase, the activators of plasminogen and hence fibrinolysis. It promotes invasion and metastasis of cancer cells and correlates with poor prognosis in breast cancer (M. Duffy, 2004; M. J. Duffy et al., 2014; Wei et al., 2019). Similarly, ITGB1 (integrin beta-1), a membrane receptor for e.g. collagen, fibronectin, fibrinogen and others, is strongly upregulated in CAS. ITGB1 is involved in cell adhesion and recognition in different kind of processes such as tissue repair, metastatic diffusion of tumor cells, immune response, embryogenesis, and hemostasis. It is known to correlate with low survival rates of triple negative breast cancer. Finally, as it can regulate the store-operated calcium influx, ITGB1 also regulates cancer cell migration (Klahan et al., 2016; Sun et al., 2018).

In essence, the results of our proteomic analysis of subsections of FFPE tissue yields detailed insight into the proteomic landscape of changes occurring during stromal reprogramming in mCA.

6.3. Substantial correlation between proteomic and transcriptomic changes during stromal reprogramming

Comparative studies have found that correlations between mRNA and protein levels in model organisms can be relatively weak and uncertain or moderately positive (Vogel & Marcotte, 2012). With respect to the entire data set, the correlation coefficient between transcriptomics and proteomics was $R=0.3626$, similar to what has been reported for other tissues before (Ghazalpour et al., 2011; Greenbaum et al., 2003; Maier et al., 2009; Popovic et al., 2018). But setting the threshold of the adjusted p-value at 0.05 increased the correlation coefficient to 0.5451. Similarly,

restricting the analysis to the top 20 targets from either the transcriptomic or proteomic dataset further increased the correlation, suggesting that the more targets are deregulated, the more consistent the correspondence between mRNA and protein levels become. This was further supported by the fact that analysis of pathway enrichment in both datasets revealed a comparable picture between transcriptomics and proteomics.

In summary, while the proteomics-based analysis of LCM-FFPE tissue yielded fewer targets than the transcriptomic approach, the protein and transcript levels showed substantial correlation, especially for the most deregulated targets and pathways.

6.4. Prognostic potential of stromal changes in canine mCA

By leveraging the relevance of canine CAS as a model of the human disease, we establish increased expression of LTBP2, IGFBP2, COL6A5, POSTN, FN1, COL4A1, COL12A1, PLOD2, COL4A2, and IGFBP7 to be strongly correlated with worse overall survival for human breast cancer patients. Validation of increased Collagen IV and Fibronectin on protein level in canine CAS further substantiates these findings. Of note, PDGFRB and FAP, two CAS markers that we have previously validated as upregulated using IHC (Ettlin et al., 2017) were also found to be upregulated using proteomics.

LTBP2 is critically involved in regulation of TGF-beta signaling (Robertson et al., 2015) and its expression is significantly elevated in human breast cancer in correlation with clinical stage and other adverse prognostic factors (Gu et al., 2018). As a regulator of PI3K signaling, the expression IGFBP2 is strongly correlated with grade of malignancy in many tumors and especially in breast cancer (Hoeflich & Russo, 2015). Collagen VI has been recently identified as driver of invasion and metastasis of breast cancer and high protein levels of Collagen VI have been found by bulk tumor proteomic analysis of more than 500 human cancers (Cescon et al., 2015; F. Chen et al., 2019; Wishart et al., 2020). Strongly elevated levels of both POSTN and FN1 are well documented in a wide variety of tumors, including breast cancer, and have been shown to promote tumor invasion and metastasis through manifold effects on different cancer hallmarks (reviewed in (Efthymiou et al., 2020; González-González & Alonso, 2018)). Similarly, Collagen IV is positively correlated with larger and more aggressive breast tumors (Ioachim et al., 2002) and has been shown to promote cancer cell invasion and migration (Xu et al., 2019). Burnier et al. revealed that increased levels of type IV collagen correlate with an increase in liver-metastasis of primary tumors such as colorectal cancer (Burnier et al., 2011). High PLOD2 expression is associated with increased mortality risk in breast cancer (Gilkes et al., 2013). Functionally, it promotes fibrillar collagen formation and thereby increases tumor stiffness and is required for metastasis. IGFBP7 expression in tumor cells has been suggested to be anti-neoplastic (Jin et al., 2020). However, when

expressed in CAS, it has growth-promoting effects on tumor cells through a paracrine signaling mechanism that can promote anchorage-independent growth of tumor cells (Rupp et al., 2015). Interestingly, IGFBP7 also binds to Collagen IV (Jin et al., 2020). The fact that on the transcriptomics level only one of the top 10 adverse genes (Amini et al., 2020) actually correlate with worse overall survival, while on the protein level there are 4 among these top 10 targets that predict worse overall survival suggests that proteomic changes could potentially be a better predictor than transcriptomic changes, at least when it comes to analyzing expression of stromal genes in bulk tissue. This might be due to the fact that RNAseq data usually turns back many more differentially expressed targets than proteomics analysis (in this study we detected 13% of all transcripts on the proteomic level). Important transcriptomic changes in single targets are therefore more likely to be 'overshadowed' by other targets that might not have anything to do with overall survival in contrast to the relatively 'smaller' proteomic dataset. Another possibility is, that proteomic detection could be biased towards the most abundant proteins, which might also hold more biological relevance. While it's currently not possible to unequivocally answer this question, this clearly further underlines the point that transcriptomic and proteomic changes are not always in direct concordance and that proteomic profiling of distinct tissue compartments can provide novel insight into biological questions that go beyond validation of RNAseq data.

Hence, by providing validation of the deregulation of these previously identified differentially expressed transcripts on protein level, our proteomic analysis of CAS reprogramming further strengthens the role of these targets as disease-modulating stromal components with implications for breast cancer in both humans and dogs.

6.5. Conclusion

In conclusion, the data presented in this thesis demonstrate the feasibility to identify proteins from microdissected FFPE sections of patient samples. With regards to the main aims of this thesis, by revealing the detailed proteomic landscape of stromal reprogramming, I was able to shed light on the proteomic changes that occur in CAS from canine mCA, thereby addressing Aim 1. Furthermore, addressing Aim 2, comparative analyzes between proteomic and transcriptomic data from the same patient samples revealed the extent of correlation between proteomic and transcriptomic changes in these tissues. Finally, with regards to Aim 3 of this thesis, by translating our findings to datasets of human tissues, we were able to identify several deregulated proteins with prognostic value for human patients. Taken together, these results have the potential to contribute to a better understanding of the involvement of stromal genes in development and progression of canine and human breast cancer.

6.6. Outlook and potential future research directions

In the future, the results presented in this thesis will serve as a starting point for mechanistic follow-up studies to further understand the role of the stromal genes that are strongly deregulated between normal stroma and CAS in development and progression of both canine and human mCA.

To do so, one can envisage the use of a multitude of possible approaches ranging from *in vitro* cell culture studies to *in vivo* experiments involving e.g. rodent models of tumor development. With respect to *in vitro* cell culture systems, mechanistic interrogation using indirect or direct coculture models of stromal cells and cancer cells can be used to dissect the effect of the targets in question on tumor cell growth, motility and invasiveness. In such settings, loss- and gain-of-function approaches using siRNA, shRNA and/or CRISPR-Cas9 in stromal cells can be used to address the effect of depletion or overexpression of the target on cancer cells. Indirect cocultures would require transfer of conditioned medium from one cell type to the other to observe cancer cell behavior, while in direct coculture systems, both cell types would be grown together in direct contact. Direct coculture systems include spheroids, organoids, and organotypic tissue models and are likely to be better models of the real *in vivo* situation than 2D-culture based assays (Jenei et al., 2011; Kim et al., 2020; Yakavets et al., 2020). One of the latest techniques to gain mechanistic insight into different processes in the microenvironment regarding cell-cell and cell-stromal interactions, is 3D bioprinting. In 3D bioprinting, so called bioinks are layered by computers to generate a viable 3D construct. The materials of bioinks differ from scaffold-based and scaffold-free bioinks. Scaffold-based bioinks, the most typical type, consist of cells, hydrogels such as agarose or collagen type I, decellularized matrix components, and microcarriers which allow cells to attach and grow and therefore expand (Hospodiuk et al., 2017; Khoshnood & Zamanian, 2020; Y. Yu et al., 2016). 3D bioprinting is already used to study the biology and drug response in breast cancer, using human cancer cells (Bahcecioglu et al., 2020; Belgodere et al., 2018; Reid et al., 2019; Y. Wang et al., 2018). In contrast, in 2D culture methods, cells grow on rigid materials like glass or polystyrene without a physiological microenvironment, those stromal proteins cannot be observed to the same scale.

As the microenvironment is viewed as an interesting target for therapy (Hirata & Sahai, 2017; T. Wu & Dai, 2017), more detailed insight into the interplay between tumor cells and the surrounding stroma is highly needed. If validated mechanistically, the novel targets identified in this thesis have the potential to inspire future therapeutic approaches to help human and veterinary patients with cancer in general, and breast cancer in particular.

7. References

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