Differences in Plasticity and Expression of Developmental Genes in Canine Mammary Carcinomas and Sarcomas

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Abstract

Mammary tumors are the most common type of tumors affecting female dogs. There is a remarkable heterogeneity among mammary tumors and the underlying cause is poorly understood. The aims of this thesis were, firstly, to investigate whether tumor cells show plasticity and thereby get insight into how heterogeneity arises and, secondly, to identify embryonic developmental genes expressed in the tumors. A comparison between sarcomas and carcinomas in these respects was of central importance for this investigation

Primary tumors, an osteosarcoma, a spindle-cell tumor, and a simple carcinoma were cloned and resulting clones were subcutaneously injected in mice, to form experimental tumors. By following the expression of lineage markers in the primary tumors, the clones *in vitro*, and in the experimental tumors, it was found that the sarcoma clones showed distinct signs of plasticity, and that the carcinoma clones transformed in line with an epithelial to mesenchymal transition.

The expression of genes coding for the developmentally important bone morphogenetic proteins was studied in the clones from the tumors described above. Further, three groups of tumors, i.e., osteosarcomas, fibrosarcomas and simple carcinomas were studied using gene expression profiling technology. Thereby, extensive expression of homeobox genes, a group of important developmental genes, was identified. Sarcomas displayed the most prominent expression of developmental genes, both for the number of genes expressed and the levels of expression for the respective genes.

Interestingly, many homeobox genes expressed in the sarcomas have been linked to craniofacial development, a process dependent on neural crest contribution. In line with this, cell markers for neural crest derived tissues were also expressed in the sarcomas, including tooth, nerve and glial markers. The results show that sarcomas are more plastic and have a more diverse gene expression than carcinomas. Heterogeneity seen in sarcomas of the mammary gland may be related to the expression of homeobox genes.

Keywords: Canine mammary tumors, plasticity, bone morphogenetic proteins, homeobox transcription factors, craniofacial, neural crest.

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wensman, H., Flama, V., Pejler, G., and Hellmén, E. (2008). Plasticity of Cloned Canine Mammary Spindle Cell Tumor, Osteosarcoma and Carcinoma cells. *Veterinary Pathology* 45:803-815.

II Wensman, H., Heldin N-E., Pejler, G., and Hellmén, E. (2009). Diverse Bone Morphogenetic Protein Expression Profiles and Smad Pathway Activation in Different Phenotypes of Experimental Canine Mammay Tumors. *In manuscript.*

III Wensman, H., Göransson, H., Leuchowius, K-J., Strömberg, S., Pontén, F., Isaksson, A., Rutteman, G.R., Heldin N-E., Pejler, G., and Hellmén, E. (2008). Extensive Expression of Craniofacial Related Homeobox Genes in Canine Mammary Sarcomas. *Breast Cancer Research and Treatment* Dec 2, [Epub ahead of print].

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Abbreviations

BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
CGH	Comparative genomic hybridisation
EMT	Epithelial to mesenchymal transition
ERBB	Erythroblastic leukaemia viral oncogene homologue
FGF	Fibroblast growth factor
GLI	Gli-Kuppel family member
HPA	Swedish human protein atlas project
IGF	Insulin-like growth factor
IHC	Immunohisto chemistry
NMT	Non malignant mammary tissue
PCR	Polymerase chain reaction
PLA	Proximity ligation assay
PTHLH	Parathyroid hormone-like hormone
PTHR1	Parathyroid hormone receptor
TGF-β	Transforming growth factor beta
TMA	Tissue microarray

Introduction

The mammary gland, an organ tightly associated with motherhood and the outermost feminine, is a complex organ with important function. Normally cells of the mammary gland divide, differentiate and die in connection with the cycling of the estrous cycle and pregnancies, but sometimes the tight control over a cell is lost and result in malignancy. Breast tumors are among the most common tumors for both women and female dogs (Ghafoor *et al.*, 2003; Lana *et al.*, 2007). In this thesis the focus will be on mammary tumors of the dog.

The mammary gland, structure and development

Structure

The mammary gland is like a tree. At the tips of the branches there are glands ready to proliferate, differentiate and produce milk in the case of pregnancy. Normally, the glands are in a resting state only submitted to minor proliferation/apoptosis as the organ prepares for a possible pregnancy with every estrous cycle. From the glands run ducts that collate with other ducts with a final purpose to collect and bring the milk to the nipple (Figure 1a).

The epithelium lining the mammary ducts and glands is a compound epithelium consisting of ductal or luminal epithelium enclosed by a myoepithelial meshwork. The myoepithelial layer (Figure 1b) can contract in response to oxytocin stimulation and has an important function in the release of milk (Gudjonsson *et al.*, 2005)

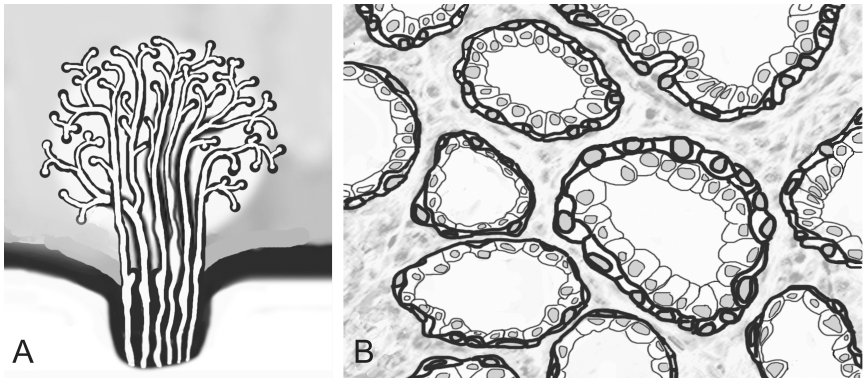

Fig. 1. The duct system of the canine mammary gland A. Section of the mammary gland tissue visualizing the compound epithelia illustrated by dark myoepithelial cells lining the luminal epithelial layer B.

Development

The processes required to induce mammary bud development (epithelial migration, change in cell adhesion, growth, death and differentiation) are also similar to the processes involved in mammary tumor development (Howard and Ashworth, 2006). The mammary gland is the result of a proliferation and ingrowth of the embryo ectoderm during development. Pluripotent epidermal cells are directed in specific lines of differentiation and a specialized structure forms (Howard and Ashworth, 2006). There are several structures in embryo development such as hair follicles and teeth that form in the same way as the mammary gland, from a skin appendage, and there are common features in the development (Mikkola and Millar, 2006). A number of common genes are expressed in hair follicle, and teeth as well as in mammary gland, and deletion of some genes affects development of all the epidermal appendages (Hennighausen and Robinson, 2001). Among the genes that have been identified as crucial in early mammary gland development are the families of homeobox and bone morphogenetic protein (BMP) genes, two families of important developmental genes that will be in focus in this thesis (Cho *et al.*, 2006; Phippard *et al.*, 1996). In addition fibroblast growth factor and Wnt signaling has been identified as very important for mammary gland development (Pispa and Thesleff, 2003). The embryonic mammary gland development is highly dependent on interactions between the epithelium and the underlying mesenchyme. Signals from the surrounding extracellular matrix promote branching morphogenesis into the mammary fat pad during normal mammary development (Fata *et al.*, 2004; Robinson, 2007)(Figure 2). Experimental

models have contributed largely to the knowledge of mammary development. Murine mammary epithelial buds can be isolated and transplanted into fat pads cleared of epithelial elements. This model enables studies of gene function in the mammary gland with genes that otherwise for example would have been embryonically lethal (Howard and Ashworth, 2006; Jackson-Fisher *et al.*, 2004; Robinson, 2004)

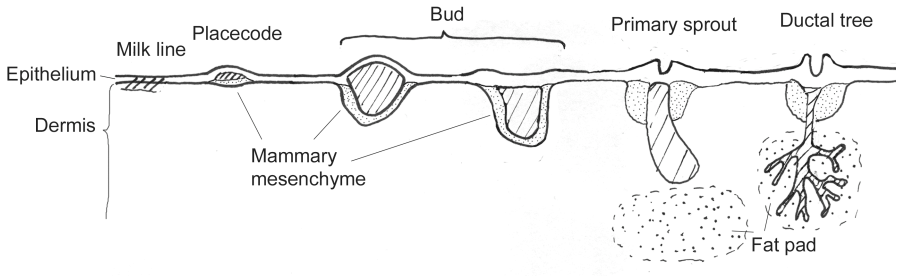

Fig. 2. Embryonic mammary gland development. The development is, in contrast to the adult developmental stages, hormone independent. To date, several regulators have been identified: Milk line formation is dependent on; fibroblast growth factor 10 (FGF10), fibroblast growth factor receptor 2 (FGFR2), WNT10b/6, neuregulin 3, Erythroblastic leukaemia viral oncogene homologue 2/4 (ERBB2/4), TBX2/3 and Gli-Kuppel family member 3 (GLI3). Placecode; WNTs, FGFs, BMP4, TBX2/3 and Lef-1. Mammary bud epithelium; Parathyroid hormone-like hormone (PTHLH), Insulin-like growth factor 1 (IGF1), BMP4, Lef-1, MSX1/2, TBX3 and GLI3. Mammary bud mesenchyme; Parathyroid hormone receptor 1 (PTHR1), BMP4, BMPR1A and MSX2. (Robinson, 2007). There is less information on later developmental stages, which might be because the primary mammary sprout is hard to distinguish from hair follicles, when they have formed. Another reason might be that factors have different roles at different stages of development. Impairment at an earlier developmental stage will obscure a role later in development.

The prenatal development of the mammary gland is hormone independent and the gland is in a resting state until hormone levels rise at puberty (Robinson, 2007). It is a special organ in the sense that it is not fully developed before birth and that it is dependent on distant hormonal control for its terminal development.

Cell markers

In order to study the mammary gland, both during normal function as well as in disease, it is important to be able to identify the different cell types comprising the gland and surrounding tissues, by using cell type-specific markers. There are a number of such markers that have been used over the years and new markers for especially mammary progenitors and stem cells

are regularly identified (Behbod *et al.*, 2006; Dontu *et al.*, 2003; LaBarge *et al.*, 2007; Shackleton *et al.*, 2006; Woodward *et al.*, 2005). The major components of the mammary gland, for example the luminal epithelium and the myoepithelial cells, can be identified by the expressed filaments. The majority of the markers used in Paper I fall into this category. Examples of epithelial markers are cytokeratins of which there are more than 20 different types with different expression pattern in myo- and luminal epithelium (Asch and Asch, 1985; Upasani *et al.*, 2004). Other examples of filament markers are vimentin (expressed in mesenchymal cells), neurofilament, smooth muscle actin and skeletal myosin. The expression of filaments can be used when characterizing tumors and tumor cells with immunohistochemistry (Destexhe *et al.*, 1993; Hellmen and Lindgren, 1989; Hellmen *et al.*, 2000).

Canine mammary tumors

Incidence and breeds

Mammary tumors are the most common form of tumor affecting female dogs (Bonnett *et al.*, 2005; Lana *et al.*, 2007). A fact that is becoming more and more clear is that the frequency of mammary tumors varies among different breeds of dogs. To date, several large studies have addressed the incidence of mammary tumors, and breeds found to have high risk include English springer spaniel, Doberman, Cocker spaniel and Boxer (Dobson *et al.*, 2002; Egenvall *et al.*, 2005; Moe, 2001). The geographical location of the studied dog populations seems to affect the incidence rates of breeds. In this thesis, the studied tumors come from different breeds. The biology of the mammary tumors, i.e., the type and malignancy, are so far thought to be the same among breeds, although there is a study claiming that small sized breeds are less prone to acquire malignant tumors (Itoh *et al.*, 2005).

A method that substantially reduces the risk of mammary tumors is ovariohysterectomy at an early age. The procedure of neutering only has effect if it is done early in the dog's life; before the first estrus gives the best protection with a risk of 0.5% of developing mammary tumors. If the procedure is done before the second estrous the risk is 8%. After the second estrus and any estrus thereafter the risk of developing mammary tumors is 26% (Schneider *et al.*, 1969). The findings thus suggest that development of mammary gland tumors is dependent on the endocrine environment, the

first few estrus cycles seem critical, and that it can be manipulated by neutering.

Biology of tumors

Tumors of the mammary gland are very heterogeneous in terms of morphology and biological behavior. The majority of the tumors have epithelial morphology, i.e. carcinomas and a minority, 4-8%, are of mesenchymal morphology, i.e. sarcomas (Bostock, 1986; Hellmen *et al.*, 1993). There are several types of both carcinomas and sarcomas. Carcinomas are, for example, further divided into simple and complex carcinomas where simple carcinomas are of tubulopapillary, solid or anaplastic type and complex carcinomas have both luminal and myoepithelial cells as part of the tumor. Examples of sarcomas are fibrosarcomas and osteosarcomas (Misdorp *et al.*, 1999). The origin of mammary tumors has for long time been under debate, especially the origin of cartilage and bone is questioned. Several alternative originating cells have been suggested, including myoepithelial cells (Espinosa de los Monteros *et al.*, 2005) and pluripotent stem cells (Gartner *et al.*, 1999; Hellmen *et al.*, 2000), with these alternatives also being considered in human breast cancer research (Leibl *et al.*, 2005; Leibl and Moinfar, 2006; Polyak, 2007; Reis-Filho *et al.*, 2006; Sorlie *et al.*, 2001).

Comparative aspects

The question is whether research on canine mammary tumors can contribute to the advancement of knowledge and ultimately the treatment of breast tumors affecting women. Research on spontaneous canine mammary tumors has been performed for many decades but still canine tumors are not very well established as a model for human malignancies. A major step forward for canine research was the release of the canine genome in 2005 (Lindblad-Toh *et al.*, 2005) which opened the door for advanced canine medical research. Importantly, the biology of canine mammary tumors and human breast tumors are very similar in many aspects. Dogs are large and relatively outbred compared to laboratory animals and the genetic differences among breeds offer a cross-sectional value higher than that in studies of inbred rodent models (Paoloni and Khanna, 2008). The background genetic diversity in dog breeds is similar to that seen in human populations based on single nucleotide polymorphism frequency (Lindblad-Toh *et al.*, 2005). Genes involved in tumor development (MET, IGFIR, KIT, mTOR and P53) have been compared between species and the canine

genome showed greater similarities to the human DNA sequence compared to the mouse (Chu *et al.*, 1998; Hoffman and Birney, 2007; Paoloni and Khanna, 2008), an indication that the canine model can be a resource in tumor research in general. Another study suggests a comparative aspect between mixed-type tumors from human salivary and canine mammary glands (Genelhu *et al.*, 2007). There are several examples of similar biology and histology of mammary tumors between humans and dogs. For example, mammary sarcomas show the same metastasizing patterns in both species (Fong *et al.*, 1993; Gullett *et al.*, 2007; Silver and Tavassoli, 1998). Another example is a study that evaluated new classifications of human mammary tumors based on gene expression profiling data on canine specimens. The classification was applicable on canine tumors and could predict outcome (Gama *et al.*, 2008).

Mechanisms behind tumor heterogeneity

Tumor stem cells

One of the most important and useful properties of stem cells is that they can self-renew and there are striking similarities between stem cells and tumor cells regarding this property. The idea that tumors in fact may be the result of a transformed normal stem cell is still under investigation (Reya *et al.*, 2001). Tumor stem cells were first identified in the hematopoietic system (Bonnet and Dick, 1997) but it was not until recently that tumor stem cells in solid tumors were identified, including breast tumors (Al-Hajj *et al.*, 2003). There is an increasing amount of evidence suggesting that tumors contain a fraction of cells that have stem cell properties (Hermann *et al.*, 2007; O'Brien *et al.*, 2007; Singh *et al.*, 2004). There are also advancements in the techniques of isolating or enriching cell cultures of tumor stem cells. For example the combination of suspension culture with chemotherapeutics enriches tumor stem cells (Li *et al.*, 2008). Identification and isolation of the tumor stem cells is the key to the development of novel treatments and therapeutics.

Whether tumor stem cells can be generated from normal stem cells remains to be shown. Another possible way for a tumor stem cell to form is by reprogramming a differentiated cell. This way of transformation has been demonstrated recently and is a breakthrough in stem cell research. It has been shown that adult fibroblasts, by mutations of very few genes, can be

reprogrammed into pluripotent embryonic-like stem cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007). It is thereby shown that certain genes and signal pathways have a very strong impact on stem cell properties. In combination with oncogenes it is possible to keep tumor stem cells in an undifferentiated state (Ben-Porath *et al.*, 2008).

Clonal evolution

Another force that is working in tumors is clonal evolution or clonal heterogeneity. In a population of tumor cells, unique mutations will occur in single cells. The cell with the unique mutation will give rise to a cell clone and an accumulation of different mutations will cause the tumor cells to gain or lose properties that make them different from each other. The force of evolution will make clones with beneficial mutations the dominating cells in the tumor. Clonal evolution can explain heterogeneity within a tumor as well as the finding that cancer stem cells can differentiate and cause heterogeneity. There is substantial evidence for both clonal evolution and the cancer stem cell model and, importantly, there are indications that the models are not necessarily mutually exclusive (Campbell and Polyak, 2007).

Developmental genes and events in tumors

Epithelial to mesenchymal transition

The cells in an epithelial tumor, a carcinoma, need to escape the tight attachment to neighboring cells to be able to move from the primary tumor and establish distant metastases. The mechanism behind this phenomenon is called epithelial to mesenchymal transition (EMT) (Thiery, 2003; Thiery and Sleeman, 2006). In early embryonic development, EMT is essential and the active process behind the formation of the mesoderm (Trelstad *et al.*, 1966) followed by the development into multiple tissue types (Nakaya and Sheng, 2008). It has also a role in wound healing (Savagner *et al.*, 2005). Through developmental and tumor studies, many of the molecular actors behind EMT have been identified (Cano *et al.*, 2000; Comijn *et al.*, 2001; Hartwell *et al.*, 2006; Yang *et al.*, 2004). The EMT concept is established and well studied. Recently a new direction of the concept opened up when it was suggested that EMT generates cells with properties of self-renewing stem cells (Mani *et al.*, 2008).

Homeobox genes, tumors and metastasis

Homeobox transcription factors are among the most important families of transcription factors in embryo development. Homeobox domains were first discovered in homeotic genes of the *antennapedia* and *bithorax* complex in *Drosophila* (McGinnis *et al.*, 1984; Scott and Weiner, 1984) with the role of determining the morphologic character of the body segments. A mutation of these genes can cause bizarre malformations such as extra sets of wings or legs instead of antennae. The DNA sequence characteristic for the homeobox encodes a recognizable although very variable protein domain, the homeodomain (Gehring *et al.*, 1990; Kissinger *et al.*, 1990). The vast majority of the homeodomains are 60 amino acids long. In vertebrates they have important roles in embryo patterning (Cho *et al.*, 1991), cell differentiation and migration (Niehrs *et al.*, 1993) and have been implicated in disease and congenital abnormalities (Dobreva *et al.*, 2006). The reported number of homeobox genes in the genome varies quite significantly in the literature. Recently a thorough investigation was performed that identified 235 probable functional homeobox genes and 65 probable pseudogenes (Holland *et al.*, 2007). Homeobox genes are well organized in the genome where they are expressed in a very controlled spatiotemporal manner in development (Iimura and Pourquie, 2007) and they are important for mammary development (Chen and Sukumar, 2003). There are significantly fewer studies of developmental genes in mammary development compared to other organ systems. The reason is simply that much of fundamental developmental research is performed in Drosophila, Xenopus Zebrafish and avian (chicken) model systems. Those model systems are in many ways easier to work with than mice but they lack mammary glands. In mammary development the homeobox genes MSX1/2 have been shown to have a role in the development of the mammary bud epithelium (Robinson, 2007) and MSX2 in branching morphogenesis (Satoh *et al.*, 2007).

Homeobox genes are not only expressed in the embryo, they are also important for functional differentiation in the adult where they, for example, have been linked to milk production (Chen and Capecchi, 1999). Despite the known function of homeobox genes, the regulatory mechanisms that drive homeobox expression are poorly understood. However, in recent studies several hormones and their cognate receptors have been implicated in homeobox gene regulation and thereby embryonic development as well as in functional differentiation in the adult. Further, estradiol, progesterone, testosterone, retinoic acid, and vitamin D have been

shown to be involved in homeobox gene regulation (Daftary and Taylor, 2006).

There is an increasing interest in homeobox genes and their role in tumorigenesis and metastasis. Homeobox genes have been shown to have a role in EMT (Comijn *et al.*, 2001; Hartwell *et al.*, 2006), detachment and tumor metastasis. Homeobox genes are master regulators and have been shown to regulate many other genes. One example is SatB1 which has been shown to control the expression of many other genes by controlling DNA methylation (Yasui *et al.*, 2002) and the injection of Goosecoid into the ventral side of a xenopus embryo generates an additional body axis including head structures and notocord structures (Cho *et al.*, 1991). Both SatB1 and Goosecoid have roles in tumor metastasis (Han *et al.*, 2008; Hartwell *et al.*, 2006) by controlling genes for example associated with cell adhesion.

BMPs, tumors and metastasis

BMPs are members of the transforming growth factor beta (TGF-β) superfamily. As the name implies they are involved in the process of bone differentiation as discovered two decades ago (Wozney, 1989), but they are also involved in other processes during development and they are also important in tumor biology. The BMP signal is transduced to the nucleus via phosphorylation of the signal transducer proteins Smad 1, 5 and 8 which together with Smad 4 immediately relocalize to the nucleus and the target genes (Shi and Massague, 2003). To date, about 15 BMPs have been identified and characterized. Experiments have shown that the knockdown of many BMP genes have embryo lethal effect and in the case of BMP 2 and 4 the effects occur very early in the development (Chang *et al.*, 2002). In development, BMPs are required for the dorsoventral patterning within the neural tube, introduction of mesoderm during gastrulation and hematopoiesis (Attisano and Wrana, 2002). BMPs have also been shown to regulate the lineage decision of differentiation in neural crest stem cells (Shah *et al.*, 1996)and are known to work together with homeobox genes in mammary development (Phippard *et al.*, 1996).

In tumor biology there have not been so many studies on BMPs compared to the massive focus on the TGF-βs in the same superfamily. Recently however there is a growing number of studies implying a role for BMPs in tumors, mainly in the metastasizing process (Alarmo *et al.*, 2007; Helms *et al.*, 2005; Montesano, 2007). In canine mammary tumors, BMP-6 and BMP

receptors have been identified in myoepithelial cells (Akiyoshi *et al.*, 2004; Tateyama *et al.*, 2001) and BMP-2/4 in a mammary osteosarcoma cell line (Kawabata *et al.*, 2006).

Retinoic acid

For years vitamin A (retinol) and its corresponding metabolite retinoic acid have been known to be essential for embryo development. On the other hand, excess of these substances will result in severe congenital abnormalities that can involve the face, eye, hindbrain, limbs and urogenital system. Further, too little or too much retinoic acid can also result in serious disturbances in the development of the hindbrain and pharyngeal neural crest (Carlson, 2004). In experimental setups, retinoic acid can cause homeotic changes, i.e. an extra pair of limbs in amphibians (Mohanty-Hejmadi *et al.*, 1992). Homeobox genes are known to be targets of retinoic acid (Daftary and Taylor, 2006) and homeobox genes are likely to be the cause of the teratogen effect of retinoic acid (Williams *et al.*, 2004).

Neural crest and craniofacial development

Homeobox genes, BMPs and retinoic acid are all associated with neural crest and craniofacial development (Daftary and Taylor, 2006; Golding *et al.*, 2000; Liem *et al.*, 1995; Trainor *et al.*, 2003; Tribulo *et al.*, 2003; Williams *et al.*, 2004). Neural crest originates from cells that migrate from the lateral margins of the neural plate. The migration is induced by signals from the non-neural ectoderm and a gradient of BMP 4 and 7 (Liem *et al.*, 1995) as well as Wnt (Steventon *et al.*, 2009) is important for the induction. The very first step of neural crest formation is for neural crest cells to lose the epithelial morphology, the attachment to the neighbors, and be submitted to EMT. This step is of high interest in tumor and metastasis research and neural crest formation can offer a natural model for EMT and metastasis (Acloque *et al.*, 2008). Detached neural crest cells have the ability to move through the basal lamina of the neuroepithelium and sequentially undergo a remarkable series of migrations throughout the developing embryo (Trainor *et al.*, 2003).

Gene expression profiling of breast cancer

The relatively new technique of global gene expression analysis of primary tumors and cell lines has provided a powerful tool to find new subclasses of tumors relevant for treatment and prognosis but also to gain more knowledge of the tumorigenesis. Much effort has been put into finding a "minimal" set of genes to identify subclasses of tumors and there are examples of identified gene sets that have been successful in predicting outcome of disease (Buyse *et al.*, 2006; Fan *et al.*, 2006; Paik *et al.*, 2004; van't Veer *et al.*, 2002). Out of the very few expression array studies that have been performed on canine tumors, there is one example of expression analysis in canine gliomas (Thomson *et al.*, 2005) and canine mammary tumor cell lines have also been subjected to expression array analysis (Rao *et al.*, 2008).

Aim of study

The overall aim with this thesis was to gain information about the plasticity of mammary tumor cells and expression of a number of genes active in normal embryo development. It was also to compare plasticity and gene expression between carcinomas and sarcomas.

The specific aims were:

• To investigate the plasticity of cloned cell lines from three primary tumors, an osteosarcoma, a spindle-cell tumor and a simple scirrhous carcinoma, by comparing protein expression patterns in the primary tumors, the cloned cell lines and in the experimental tumors formed by the cell clones.

• To investigate the mRNA expression profile of BMPs in cloned cell lines and make an attempt to correlate the profile with the phenotype of the experimental tumors formed by the clones.

• To compare the global mRNA expression patterns in three groups of tumors (osteosarcomas, fibrosarcomas and simple carcinomas) as well as to non-malignant mammary tissue (NMT). Moreover, and if possible, to identify developmental master genes i.e. genes that regulate many other genes.

Comments on materials and methods

Primary Tumors

The primary mammary tumors in Paper I and II were a simple scirrhous carcinoma, an osteosarcoma and a spindle-cell tumor. The carcinoma and the osteosarcoma came from the same dog.

When the tumors for the expression array were selected, the initial idea was to choose both mammary carcinomas and sarcomas. The carcinoma group chosen was of the simple carcinoma subtype, i.e. monophasic tumors comprising one type of neoplastic cells. The sarcoma groups chosen for the study were fibrosarcomas and osteosarcomas. All tumors were malignant.

Cell culture, cell cloning

The cloning was performed carefully to assure the origin of a clone from one cell. The experimental design in Paper I and II was dependent on one cell as the origin of one clone and failure would have hampered the interpretation of the results. The cells were plated sparsely by seeding approximately 30 cells per 10-cm cell culture dish. The following day, single cells with large distance to neighbors were marked out with a pen and later chosen.

To decrease the influence of *in vitro* clonal evolution the experiments were performed with as low passages of the cloned cells as possible in the two studies. For the establishment of the experimental tumors in Paper I and II passages 4-9 were used, with the majority around 5. That means that the cells were in culture very short time. It is also worth mentioning that the primary cell lines were cloned at a low passage (around 20).

Stimulation of cell lines with BMP-2

Cells were stimulated for 1 hour with BMP. It has been shown for the TGF-β pathway, which has Smad-4 as a common signal transmitter together with the BMP pathway, that it only takes minutes of stimulation to get a relocation of signal transmitters to the nucleus (Pinidiyaarachchi *et al.*, 2008). Hence the stimulation time was adequate and we also observed activation. As a control of the activation the anaplastic thyroid carcinoma cell line HTh 74 was used because of its known ability to respond to BMP stimulation (Heldin *et al.*, 1991).

Immunohistochemical analysis

The immunohistochemical characterization of the cell clones used in Paper I and II was performed on frozen cells and control specimens. The fixation was performed with acetone, which is adequate for many antibodies on frozen tissues.

In the following immunohistochemical analyses on formalin fixated and paraffin imbedded tissues in Paper I-III, an efficient antigen retrieval was required to expose the epitopes for the antibodies. A heat-mediated antigen retrieval in a boiling chamber is very efficient and the buffer and the boiling time can be optimized for every antibody. When the conditions for the antibodies in this thesis were optimized different buffers were tested and boiling times were keep constant to reduce the variable parameters.

The immunohistochemical analyses of bone sections were extremely difficult technically. The first difficulty lay in the fact that bone is very hard to section. It needs to be decalcified which may influence the morphology and epitopes if this is too harshly performed. After decalcification, perfect

sections are nevertheless difficult to obtain due to the hardness of the material. The largest problem is however that bone and nuclei in the bone area detach from the slide in the heat-mediated antigen retrieval process. Specially coated slides (Super Frost Gold) were used to overcome this problem, but still some detachment occurred.

The antigen retrieval method is highly dependent on the antibody it is used for. For collagen type I and II antibodies (Paper I), it was adequate to treat the deparaffinized sections with pepsin in order to reveal the epitopes. Those slides were therefore never subjected to heat mediated antigen retrieval, hence the excellent morphology of those immunohistochemical analyses.

For immunohistochemical analysis, one of the most important steps is the selection of the antibody. Commercial antibodies raised against canine proteins are extremely rare. However, it is becoming more common that dog is included among the species antibodies are tested against by the companies. The release of the canine genome also makes it possible to do an alignment between the peptide the antibody is raised against, usually of human or murine origin, and the corresponding canine sequence. That is no guarantee of good performance but it is a hint of how the antibody will perform on canine tissues.

With all the antibodies, both positive and negative controls were used. For neurofilaments, the stroma around the tumor provided peripheral nerves that could be used as internal positive controls.

The antibodies generated within the Swedish Human Protein Atlas Project (HPA) (Uhlen *et al.*, 2005) (Paper III) were raised, in rabbits, against peptides of human origin. The antibodies were generated using an antibody-based proteomics approach (Uhlen and Ponten, 2005). Antibodies were affinity purified on columns coated with the antigens they were raised against. The strength of the HPA antibodies is the careful validation with Western blot and IHC on tissue microarrays (TMA) with a high coverage of tissue types and tumors. In that way protein expression patterns and antibody performance can be analyzed in detail.

TMAs are an efficient way of analyzing many tissues at the same time by IHC. The method can significantly increase the throughput of an otherwise labor-intense and time-consuming method as IHC (Kononen *et al.*, 1998).

In the second paper in this thesis, a TMA was constructed to overcome the problem of performing IHC on bone tissue. The osteosarcomas were possible to section although the resulting sections could be more or less intact. The large obstacle to overcome was that the bone tissues present in the osteosarcomas frequently fell off the slide in the antigen retrieval process. A TMA was constructed from the experimentally osteosarcomas (Paper II) and the punches were taken in cell-dense areas of the tumors without bone tissue. This approach was successful and the experimental osteosarcoma TMA could thus be used for IHC analyses.

DNA index

The DNA content (DNA index) of the clones (Paper I) was studied with flow cytometry. Despite being a crude method, all the clones had, before the injection in nude mice, single peaks with measurable differences in DNA content between the clones, i.e. if two clones had been mixed or there had been a clonal evolution (with the same magnitude of the loss or gain of DNA as between the clones) there would have been two (or more) G1 peaks.

Tumorigenicity in nude mice

The generation of experimental mammary tumors was performed with female Balb/c nu/nu mice. The influence of hormones to the genesis of mammary tumors is a factor to take into account when designing the experiments. Therefore, only female mice were used to reduce the influence of unknown variables in our experiments.

Ribonuclease Protection Assay (RPA)

This method is quantitative and sensitive. In brief total RNA from the different clones were hybridized to probes of different length designed to detect mRNAs of BMP-1, -2, -3, -3B, -4, -5, -6, -7, -8A and -8B, including L32 and GAPDH as internal controls. The ^{32}P-labeled antisense RNA probes in this method vary considerably in length for the different

BMP genes, from 125 to 381 nucleotides. After hybridization, ribonuclease was added to degrade unprotected, unhybridized single stranded RNA. The differences of the probes in length are necessary for the separation on an acrylamid gel and identification of the different bands. The probe set used (Paper II) was designed for mouse and therefore the human thyroid carcinoma cell line Kat-4 was used as an extra positive control in addition to the positive mouse control provided with the RPA kit. The localization of the canine BMP bands on the gel was directly comparable with those of the mouse control and the BMPs expressed in the human cell line Kat-4. The interpretation of the controls was that the probes gave a reliable identification of expressed canine BMP genes. The relatively long probes probably contributed to the reliability. Mismatched probes would most probably have been digested by the RNase and interpreted as (false) negative expression, a more likely scenario in inter-species hybridizations and RPA than a false positive match.

RNA preparation and quality validation for expression analysis

The quality of the RNA for an expression array analysis is essential. Therefore, considerable effort was put into assuring as good quality of the RNA from the primary tumors as possible. The vast majority of tested tumors did not qualify. The RNA was first prepared by pulverizing the tumor specimen under liquid nitrogen followed by addition of Trizol. The quality was first measured with an Agilent 2100 Bioanalyzer and a second time after the RNA purification on Qiagen mini columns. The concentrations were measured on a NanoDrop ND-1000 spectophotometer after purification.

The Affymetrix method

Affymetrix GeneChip® expression arrays (Canine Genome 2.0 Array) were used, a high-resolution array produced with a photolithography technique. The technique of production differs from other expression array platforms and is adapted from the semiconductor industry. One by one the nucleotides are added to the chip and 25-mer oligonucleotide probes are built. About 15 unique probes are designed for every gene and every probe

has millions of copies (Dalma-Weiszhausz *et al.*, 2006). One sample is used for one array hybridization and result in a quantitative figure on gene expression since the number of probes is controlled.

Result and discussion

In this thesis, Paper I and II are related and investigate three primary mammary tumors (a spindle-cell tumor, an osteosarcoma and a carcinoma), the cell clones generated from the primary tumors (referred to as the *in vitro* step) and the experimental tumors generated from the clones, *in vivo* step (see Fig. 3). Paper I mainly focuses on the plasticity by following the expression of cellular markers in the primary tumors, the cloned cell lines and eventually in the experimental tumors generated from the clones. Paper II focuses on the expression of BMP genes, BMP-6 protein expression and the activation of the BMP signaling pathway.

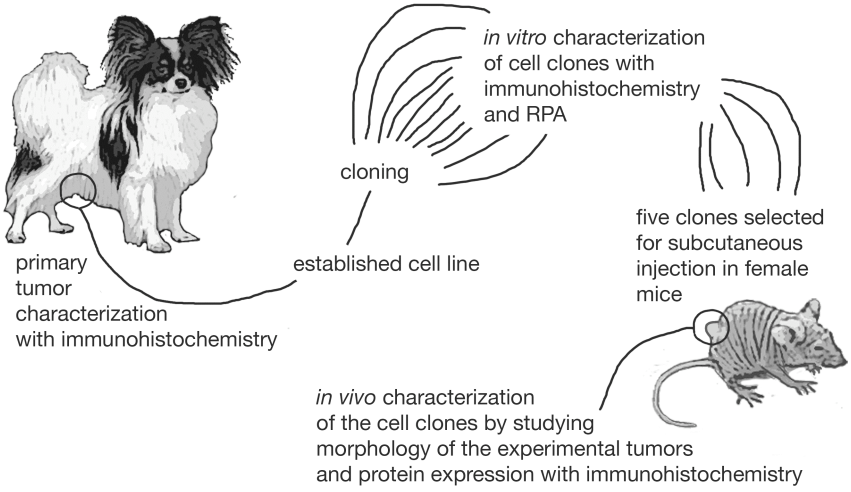

Fig. 3. Experimental outline for Paper I and II

The experimental design is unusual; it generates considerable information from a small number of primary tumors. The common way of studying clonality in solid tumors is, for example, to use polymerase chain reaction (PCR) or comparative genomic hybridization (CGH) to study differences in mutations in different parts of the tumor (Jones *et al.*, 2000; Lien *et al.*, 2004). In such an approach, DNA is extracted from fixed and thereby dead tissue. The advantage of the design used here is that living cell clones were studied.

Plasticity or clonal evolution? (Paper I)

A very interesting observation was that three different clones from the primary osteosarcoma did not express any of the cellular markers tested for *in vitro*, but the same clones formed highly differentiated experimental osteosarcomas. The clones from the spindle-cell tumor also formed experimental tumors with foci of highly differentiated bone. The primary tumor did not have any bone formation. By following the expression of filaments in primary tumors, clones *in vitro* and in the experimental tumors, the conclusions were that spindle-cell and osteosarcoma clones showed plasticity, i.e. the clones had the ability to turn off and on genetic programs. For example, vimentin expression may have been turned off in three different osteosarcoma clones (clone 1, 2 and 7) when they were cloned and cultured. Nearly all of the tumor cells in the primary osteosarcoma were vimentin positive. In cell culture, all cells in the three clones were negative to all markers tested, an indication of very immature cells (Jackson *et al.*, 1980; Paulin *et al.*, 1980), but all three clones were still able to form tumors and form bone in experimental tumors, accompanied by an onset of vimentin expression. The findings that genetic programs apparently can be turned on/off in a controlled way and that multiple clones performed in the same way favor plasticity as the underlying force.

Collagen type I and II expression showed an unexpected expression pattern in the experimental osteosarcoma tumors where collagen type II, normally found in cartilage, was expressed in the highly differentiated foci of bone in the experimental osteosarcomas. On the other hand, collagen type I, normally expressed in bone tissues, was not expressed in the highly differentiated foci of bone in the same tumors. Both collagen type I and II were expressed in the experimental osteosarcomas in the areas of

undifferentiated and less differentiated bone tissue surrounding the foci of bone. It has been reported that during chondroid differentiation coexpression of collagen I and II can occur and that the expression profile change during the course of differentiation (Hegert *et al.*, 2002; Poliard *et al.*, 1999).

The third primary tumor, the carcinoma, gave rise to clones where the majority (4/5) lost the expression of keratin, an epithelial marker, *in vitro*. The experimental tumors from the carcinoma also showed low expression of keratins and some tumors lacked keratin expression. The morphology was spindle-cell (fibroblast) like, i.e., showed no sign of epithelial morphology as the primary tumor. The conclusion was that carcinoma clones formed tumors in line with an EMT.

Another possible explanation for our results was clonal heterogeneity or clonal evolution (Campbell and Polyak, 2007). According to such a model, the acquirement of mutations during cell culture could generate heterogeneity in our experimental tumors. However clonal evolution was not considered to be the cause of the heterogeneity in our experimental setup for the following reasons: Considerable effort was put on minimizing the influence of clonal evolution by using the cell clones at a low passage number. Further, bone formation is the result of complicated genetic programs and not likely to be the result of mutations by chance. Bone formation was seen in a number of tumors from 5 different clones (3 osteosarcoma clones and 2 spindle-cell clones) and that seems more than a coincidence and likely the result of plastic tumor stem cells.

BMP expression and tumor phenotype (Paper II and III)

The mRNA expression profile of a number of different BMPs was studied in the cell clones from the same three primary tumors as above. In addition, Smad activation and expression of BMP-6 protein were studied in the experimental tumors. A high expression of several BMPs was found not only in the osteosarcoma clones but, unexpectedly, also in the spindle-cell clones. The primary spindle-cell tumor displayed no bone formation and hence it was expected that the clones derived from the tumor would express low levels of BMPs. However expression of BMP-2,-3B,-4,-5,-6,-7 and -8 was found in the ostesarcoma- and spindle-cell clones, but the expression

patterns in the different clones varied significantly. Further, the carcinoma clones showed low but clearly detectable expression of BMP-2 and -4.

As in the first study it was important to work with low passages of the clones to ensure that the expression patterns found represented that of the primary tumor. There is increasing information on BMPs in relation to tumor growth and invasion and in most cases BMPs are expressed without causing bone formation (Alarmo *et al.*, 2007; Helms *et al.*, 2005). In breast cancer research, BMPs are studied and associated with metastasis to bone, which is relatively common in human breast cancer (Misdorp and den Herder, 1966; Pratap *et al.*, 2006). In canine mammary tumors, on the other hand, metastasis to bone is uncommon (Hellmen and Svensson, 1995; Misdorp *et al.*, 1999). BMPs were also expressed in the carcinoma clones (Paper II) but at much lower levels. In development, BMPs have pleiotrophic roles and are essential in, for example, neural crest specification (Trainor *et al.*, 2003; Tribulo *et al.*, 2003) and dorsal differentiation of neural plate cells (Liem *et al.*, 1995). It is possible that BMPs are active in a number of tumors but only in a minority does the expression result in osteosarcoma formation. In our experimental setup it was also shown that some clones with high expression of BMPs were unable to form tumors in the mice (CMT-U309, clone 1 and 2). The conclusion drawn was that BMP expression was not correlated to tumorigenecity.

In an attempt to correlate the BMP expression patterns of the clones *in vitro* and the phenotype of the formed experimental tumors, the results suggested that BMP-6 was needed for bone formation. The clearest example was the osteosarcoma clone 6 that had very high expression of BMP-4 but no expression of BMP-6 and formed spindle-cell tumors in contrast to the other osteosarcoma clones. BMP-6 has in several studies been linked to bone formation in both development and in tumors (Akiyoshi *et al.*, 2004; Jane *et al.*, 2002; Kang *et al.*, 2004; Kugimiya *et al.*, 2005; Sammons *et al.*, 2004). Further, activated (phosphorylated) Smad 1/5 was studied to see whether the BMP pathway was activated in the experimental tumors, in an attempt to evaluate the relevance of expressed BMPs in the clones. The analysis of Smad 1/5 activation supported the importance of BMP-6 since the Smad 1/5 pathway was active in experimental tumors derived from BMP-6 mRNA expressing clones. It was also shown that clones could respond to BMP stimulation and result in Smad 1/5 phosphorylation; in other words the clones should be capable of autocrine stimulation. The presence of BMP-6 protein was verified in the experimental tumors that

formed bone. It was thereby shown that the amount of protein expressed corresponded to the level of mRNA expressed in the respective clone, i.e. the osteosarcoma clone with very low BMP-6 mRNA expression had low expression of BMP-6 protein. Surprisingly, the experimental carcinoma tumors expressed BMP-6 protein. On the other hand, the tumors showed very little Smad 1/5 activity and therefore most likely, low levels of BMP signal had transduced to the nucleus and target genes.

BMPs were also shown to be upregulated in the ostosarcomas (Paper III). BMP-2, -4, -5, and -6 showed all high expression in osteosarcomas. This supports the importance of BMPs for the osteosarcoma phenotype. Further, BMP-2 was also expressed in two fibrosarcomas and in the carcinoma that was of basal cell type. That observation supports the RPA analysis where BMP-2 was expressed by carcinoma and spindle-cell clones. BMPs are among the known inducers of EMT (Kiefer *et al.*, 2008) and therefore can be thought to influence the ability of a tumor to metastasize.

Craniofacial and neural crest signature (Paper I and III)

The third study in this thesis is a gene expression study where the global gene expression was investigated in a number of primary mammary tumors; osteosarcomas (n=5), fibrosarcomas (n=6), simple carcinomas (n=7) and normal mammary tissues were used as controls (n=4). Affymetrix GeneChip® expression arrays were used and the expression data showed signs of high quality and reliability for the following reasons: Firstly, the tumor groups clustered well together when the data were subjected to unsupervised clustering. Secondly, the sarcomas clustered together and carcinomas clustered next to the normal mammary tissue controls with the expression of epithelial genes as common feature. Further, the tumor groups were compared by supervised clustering and a high number of tissue-specific genes were expressed in the respective group, i.e. bone related genes in the osteosarcomas, other mesenchymal and cell-matrix adhesion genes in the fibrosarcomas and epithelial genes in the carcinomas. It could therefore be stated that the data revealed a good correlation between gene expression profile and histological classification.

It was observed that several homeobox transcription factors were among the most differentially expressed genes when comparing the tumor groups (carcinomas vs sarcomas) and this called for a closer look into the expression

of homeobox genes, an important group of developmental genes that includes several mastergenes. A supervised clustering of all the homeobox genes present on the array revealed that the sarcomas, especially the osteosarcomas, expressed many homeobox transcription factors. To our surprise, two carcinomas (203B and P208) expressed homeobox genes and thereby clustered among the sarcomas. However, these two carcinomas turned out to be special, in that they were shown to be a lipid-rich carcinoma and a basal cell carcinoma, respectively.

Interestingly, a great number of the homeobox genes identified in this study, for example Goosecoid (Yamada *et al.*, 1995), SatB2 (Dobreva *et al.*, 2006), MSX1 and MSX2 (Hosokawa *et al.*, 2007; Ishii *et al.*, 2005), DLX5 and DLX6 (Robledo *et al.*, 2002), paired related homeobox 1 (Lu *et al.*, 1999) and SIP1/ZEB2 (Van de Putte *et al.*, 2003) have previously been linked to craniofacial bone development; in total 16 homeobox genes expressed in the sarcomas have been found to be active in craniofacial development (Table 1) and craniofacial bones are dependent on neural crest to develop.

Table 1. Homeobox genes expressed in sarcomas (published as supplementary material to Paper III). The order of appearance is the same as in Fig 2 in Paper III

Gene name	Role in craniofacial development
DLX-5	Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation.(Levi *et al.*, 2006) The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development (Robledo *et al.*, 2002)
Paired-like homeodomain transcription factor 2 (PITX2) isoform c	Genetic dissection of Pitx2 in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. (Liu *et al.*, 2003)
Paired related homeobox 1	prx-1 functions cooperatively with another paired-related homeobox gene, prx-2, to maintain cell fates within the craniofacial mesenchyme. (Lu *et al.*, 1999)
SIP1/ZEB2	Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome. (Van de Putte *et al.*, 2003)

MSX2	TGF-beta mediated Msx2 expression controls occipital somites-derived caudal region of skull development. (Hosokawa *et al.*, 2007) Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. (Ishii *et al.*, 2005)
Homeobox DLX-6	The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development (Robledo *et al.*, 2002)
Homeobox BarH-like 1	Cloning, characterization, localization, and mutational screening of the human BARX1 gene. (Gould and Walter, 2000)
MSX1	Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. (Ishii *et al.*, 2005) Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation. (Levi *et al.*, 2006)
Homeobox A2	Temporal requirement of Hoxa2 in cranial neural crest skeletal morphogenesis. (Santagati *et al.*, 2005)
SatB2	Satb2 haploinsufficiency phenocopies 2q32-q33 deletions, whereas loss suggests a fundamental role in the coordination of jaw development. (Britanova *et al.*, 2006) SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. (Dobreva *et al.*, 2006)
Paired mesoderm homeobox protein 2 (PRX-2)	prx-1 functions cooperatively with another paired-related homeobox gene, prx-2, to maintain cell fates within the craniofacial mesenchyme. (Lu *et al.*, 1999)
Goosecoid	Targeted mutation of the murine goosecoid gene results in craniofacial defects and neonatal death. (Yamada *et al.*, 1995)
LIM homeobox proten 1	Requirement for Lim1 in head-organizer function. (Shawlot and Behringer, 1995)
SIX1 (Sine oculis homeobox homolog 1)	Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice. (Laclef *et al.*, 2003)
pituitary homeobox 1 (PITX1)(hindlimb expressed homeobox protein backfoot)	Deletion of the Pitx1 genomic locus affects mandibular tooth morphogenesis and expression of the Barx1 and Tbx1 genes. (Mitsiadis and Drouin, 2008)
Homeobox A7	Craniofacial abnormalities induced by ectopic expression of the homeobox gene Hox-1.1 in transgenic mice. (Balling *et al.*, 1989)

Another group of genes related to bone formation, the BMP genes, were found to be expressed mainly in the sarcomas. Further, in a search for genes known to interact with homeobox genes, genes involved in retinoic acid signaling were identified.

Homeobox genes together with BMPs and genes related to retinoic acid signaling have important roles in early embryonic development and neural crest formation and specification (Ko *et al.*, 2007; Steventon *et al.*, 2009), including craniofacial development. Neural crest give rise to as diverse tissues as peripheral nerves, smooth muscle, cartilage, bone and teeth (Trainor *et al.*, 2003). With those kinds of genetic programs activated, it can not only explain the ability of tumor cells to form bone but also the expression of other genes like neural genes e.g., Microtubule associated protein 1A and transcription factor COE1 involved in dendritic branching and development of medium spiny neurons, respectively and glial genes like meteorin. Moreover, tooth markers like dentin, sialophosphoprotein and amelogenin are expressed. In fact, the expression of neurofilament (Paper I) can be explained by neural crest genetic programs. It is genetic programs like these that are the key to the diversity seen in mammary sarcomas.

Differences between carcinomas and sarcomas (Paper I-III)

Homeobox genes (Paper III) have roles early in development and can be considered as progenitor or stem cell markers, for example, Goosecoid (Boucher *et al.*, 2000), DLX-5 (Tonchev *et al.*, 2006) and Homeobox B4 (Chan *et al.*, 2008). BMPs (Paper II and III) are also important for stem cell signaling. It is stem- and progenitor cells that are the main targets of BMPs (Wagner, 2007). Sarcomas expressed a higher number of homeobox and BMP genes than did the carcinomas, indicating that sarcomas probably have a larger fraction of tumor stem cells than carcinomas. The results presented in this thesis (Paper I and III) indicate that sarcomas are more plastic and show more diversity in RNA and protein expression than carcinomas.

Recent studies reveal that only a few mutations can transform a fibroblast to a cell with stem cell properties (Takahashi *et al.*, 2007; Yu *et al.*, 2007) and that fact also influences the way to think about tumorigenesis and tumor stem cells. There are probably several types of cells in the mammary gland that can be transformed to a tumor stem cell. In the future, the

identification of genetic programs that are related to biological properties will be important. For example, the two carcinomas that were lipid rich carcinoma and basal cell carcinoma (Paper III) could be distinguished from the other carcinomas due to their homeobox expression that most probably determine properties of those two tumors.

Conclusions

The results in this thesis indicate that mammary sarcomas are plastic. They appear to have the ability to turn on and off genetic programs and express genes associated with progenitor/stem cells. Carcinomas also showed ability to transform, in line with an EMT, but did not have the same diversity in gene expression as the sarcomas.

The specific conclusions:

• Osteosarcoma clones can turn off and on the bone forming program, a sign of plasticity. Spindle-cell clones also formed bone and carcinoma clones performed an EMT.

• Osteosarcoma and spindle-cell clones expressed many different BMPs and BMP-6 which could be important for bone formation.

• Expression of many homeobox genes crucial in development were identified in sarcomas. The homeobox gene expression pattern can explain the heterogenous expression among sarcomas of, for example, bone, cartilage, nerve, glial and tooth markers.

Future perspectives

More knowledge tends to generate even more questions and that is the case with this thesis. There are several lines of research that could be pursued. To start with it would be interesting to design experiments and study how the homeobox genes identified in Paper III influences metastasis and survival rates. There is an increasing number of studies in which homebox genes have been identified as drivers of metastasis. Interestingly, the majority of those homeobox genes were also expressed in sarcomas (Paper III), but many of the homeobox genes expressed in the sarcomas have never been studied in tumors or in relation to metastasis. Hand in hand with this comes also the identification of prognostic markers that could be useful in the clinic to predict survival. Increased knowledge of the actors in metastasis are the basis for the development of new treatment of highly malignant tumors or even preventing tumors from establishing distant metastasis.

It would also be interesting to further investigate the differences between sarcomas and carcinomas. Perhaps it is easier to identify metastasis genes in primary sarcomas than in carcinomas due to the small number of stromal cells in sarcomas, an advantage when preparing RNA from tumor specimens. In addition sarcoma cells do not have to perform an EMT before metastasizing. There are probably lessons to be learned from sarcomas but, first, the relevance for carcinoma metastasis has to be evaluated. Are the two tumor groups dependent on the same genetic programs to metastasize or is there more than one metastasis program?

There are studies that link BMP to EMT and tumor metastasis but there are not many target genes or modulators of BMP signaling identified in that process. There is one example of homeobox connection with TGF-β signalling: the homeobox SIP1/ZEB2 regulate Smad signaling (Postigo *et*

al., 2003). One approach to test if BMP signaling is dependent on homeobox proteins would be to perform proximity ligation assays (PLA) between phosphorylated Smad 1/5 and different homeobox genes expressed in Paper III. During the preparation of Paper III an attempt was made to perform such an analysis but due to unspecific antibodies the results were not included.

Another aspect of the neural crest signature (Paper III) is that neural crest in relation to mammary development is very little studied. The probable reason is that developmental studies most often are based on non-mammalian models. The question is if neural crest cells are involved in mammary gland development in the way they are in tooth (Jiang *et al.*, 2008) or hair follicle (Sieber-Blum *et al.*, 2004) development. Teeth, hair follicles and mammary glands are all formed from epidermal appendages in a similar way and, for example, mutation of the genes MSX1/2 and Lef-1 will affect the development of all epidermal appendages including mammary glands (Hennighausen and Robinson, 2001). It is not unlikely that neural crest cells are involved in mammary gland development and contribute to the progenitor pool in the mammary. Filling in these gaps of knowledge concerning mammary gland development would be clearly beneficial for mammary tumor research.

Populärvetenskaplig sammanfattning

Brösttumörer är den vanligaste typen av tumörer både hos kvinnor och tikar, och hos hund kallas dessa juvertumörer. Det finns en stor mängd olika typer av tumörer som kan uppstå i juvret och denna mångfald har förbryllat forskare i många decennier. De flesta juvertumörer är karcinom, det vill säga de har en epitelstruktur liknande den som juvrets körtlar består av. Dessutom finns sarkom som består av stödvävnadslika celler, t.ex. ben-, brosk-, bindväv-, och muskeltumörer. Särskilt hur brosk och ben i juvertumörer uppstår har varit ett frågetecken.

På senare år har det framkommit att tumörer till viss del består av celler med stamcellsegenskaper, så kallade tumörstamceller. En egenskap som förenar en vanlig stamcell med en tumörstamcell är bland annat möjligheten att bilda dotterceller med helt andra egenskaper och med helt andra gener aktiva. Denna förändring av egenskaper kännetecknar plasticitet. Tumörstamceller skulle kunna vara orsaken till den mångfald som ses bland juvertumörer.

Syftet med denna avhandling har varit att få kunskap om tumörcellers plasticitet d.v.s. förmåga att sätta på/stänga av gener. Vidare har målet varit att försöka identifiera aktiva gener, normalt viktiga i embryonalutvecklingen, hos olika typer av tumörer. Särskilt karcinom och sarkom har jämförts.

I den första studien studerades tre tumörer, två sarkom (en bentumör och en bindvävstumör) och ett karcinom. Enskilda celler separerades ut och tilläts ge upphov till kloner. Dessa kloner studerades först i cellodling och sedan efter att de återigen bildat experimentella juvertumörer från hund under skinnet på möss. Cellmarkörer studerades, vars förekomst avslöjar celltypen, d.v.s. om det är en ben-, brosk-, muskel-, epitel- eller nervcell. Det visade

sig att när man följde förekomsten av markörer i modertumören, i cellklonerna och i de experimentella tumörerna så hade tumörcellerna förmåga att ändra förekomsten av markörer. Särskilt bentumörens celler visade egenskaper som var stamcellslika. Sarkomen var mer plastiska och visade mer mångfald än karcinomet.

Det står mer och mer klart att gener och fenomen som är viktiga i tidig embryonalutveckling även har en mycket framträdande roll i tumörutveckling. Den andra och tredje studien inriktades därför på gener som är viktiga i embryonalutveckling. Två viktiga genfamiljer studerades; bone morphogenetic proteins (fritt översatt; benutvecklingsproteiner), viktiga för såväl tidig embryonalutveckling, utveckling av bröst/juver och benbildning. En annan grupp av gener som studerades var homeobox-gener, en av de allra viktigaste gengrupperna i utvecklingen av embryot. Homeobox-gener utför planritningen och bestämmer placering av extremiteter och bildandet av flertalet organ varav bröst/juver är ett av dem. Två grupper med sarkom studerades (även denna gång bentumörer och bindvävstumörer) och de jämfördes mot en grupp karcinom. Det var sarkomen som hade flest av dessa embryonalutvecklingsgener aktiva, gener som normalt har sin verkan i och på stamceller i embryot. Dessa resultat kan tyda på att sarkom har fler tumörstamceller än karcinom.

Att sarkomen och särskilt bentumörerna hade många homeobox-gener aktiva var ett mycket intressant fynd. Med tanke på vad som redan är känt om homeobox-genernas funktion, blir slutsatsen att benet i bentumörerna bildas på samma sätt som ansiktets ben. En i sin tur viktig komponent i bildandet av ansiktets ben är neurallistceller. I embryoutvecklingen ger neurallistceller upphov till ett mycket varierat utbud av vävnad såsom nerver, tänder och pigmentceller. Mycket intressant var att neurallistgener också var aktiva i sarkomen som studerats, till exempel fanns aktiva gener som normalt finns i nerv och tand. Dessa fynd ger inblick i hur mångfalden bland juvertumörer framförallt sarkom uppstår.

I denna avhandling har flera homeobox-gener identifierats som förut inte studerats i tumörer. Tidigare har andra homeobox-gener förknippats med tumörspridning (metastasering). Framtida studier av homebox-gener kan ge mer förståelse för hur tumörer sprider sig, vilket behövs för att få fram nya behandlingar av elakartade tumörer.

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