Characterization of Canine Mammary Carcinoma using Dog-Specific cDNA arrays

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Proefschrift

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Dedicated to my Parents

Chapter 1

Aims and scope of the thesis

Mammary tumors are frequently diagnosed neoplasms in non-spayed female dogs. Ovarian hormones are very important in this context as they are involved in normal development as well as neoplastic transformations of the mammary gland. Among them progesterone is an important risk factor for canine mammary tumor development as progestins are known to induce mammary gland hyperplasia and mammary gland-derived growth hormone (GH) excess in dogs. Downstream mechanism of progesterone action in the mammary gland is far from being fully resolved. Therefore, the general aim of this thesis was to improve the understanding of molecular events involved in canine mammary tumorigenesis with special emphasis on role of progesterone.

Chapter 2 reviews the hormonal/growth factor regulation of mammary gland development. More emphasis is given to the role of progesterone, GH and Wnt signaling in mammary gland development and tumorigenesis. Relevant literature regarding canine mammary tumor prevalence, histological subtypes, prognostic factors and clinical practices is also reviewed in this chapter.

Knowledge obtained from *in vitro* and *in vivo* studies using well characterized mammary tumor cell lines contribute to a great part of knowledge on breast cancer. There are not many well characterized cell lines available for canine mammary cancer research. In **Chapter 3,** characterization of three canine mammary cell lines (CMT) using canine specific cDNA microarray is described. The development and validation of canine specific cDNA array is also elaborated in this chapter. In addition, altered gene expression/cellular signaling pathways in CMTs is discussed in relation to their biological behavior.

Microarray technology allows simultaneous measurement of expression of many thousands of genes. However, biggest challenge after acquiring such a large amount of gene expression data is to validate their biological significance. In **Chapter 4**, speculated role (in **Chapter 3**) of two genes [response gene to complement 32 (RGC32) and factor B (BF)] in tumor cell proliferation was experimentally validated. Cause of the overexpression of these genes in cell lines and their expression pattern in spontaneous canine mammary tumors was also investigated. The potential use of expression of RGC32 and BF as tumor markers is also discussed in relation to cell cycle aberrations.

Progesterone is believed to be the key risk factor in canine mammary tumorigenesis as it known to induce hyperplasia and local production of growth hormone (GH) in canine mammary gland. In **Chapter 5,** GH induction in mammary gland upon prolonged exposure progestin in ovariectomized dogs was investigated. This study confirmed the previously reported progestin-induced mammary hyperplasia (CMH) and mammary derived-GH excess in dogs. CMH obtained from this study was used to further investigate molecular changes induced progestins in canine mammary gland.

Canonical Wnt signaling is known for its pivotal role in early development, adult tissue maintenance and tumorigenesis. Activity of canonical Wnt signaling is often observed in human breast carcinomas and a recent study implicated Wnt4, a ligand of Wnt signaling as a downstream target of progesterone in murine mammary gland. In **Chapter 6,** activity of canonical Wnt signaling in progestin-induced and a spontaneous canine mammary tumor was investigated. This study also investigated the activity of canonical Wnt signaling in CMTs. A cell line with active canonical Wnt signaling can be used to screen putative Wnt signaling inhibitors with therapeutic potential.

In **Chapter 7**, progestin-induced and spontaneous canine mammary tumors (CMC) were characterized using canine cDNA microarray. Apart from identifying molecular alterations induced by progestins, this study also reiterated the oncogenic potential of progestins in canine mammary gland. Gene expression profile of CMC which was developed in this study can be used to identify new tumor markers and therapeutic targets. This data set can also be used as a classifier for large-scale microarray studies aiming to classify canine mammary carcinomas into molecular subtypes.

In **Chapter 8** the results from all chapters are summarized and discussed.

Following key objectives/questions were addressed in this thesis;

1. To develop and to analyze the usefulness of canine specific cDNA microarray using cDNA derived from canine mammary cell lines. This may identify uniquely altered cellular signaling pathways is canine mammary cell lines.

2. Detailed investigation of identified altered cellular signaling pathways in canine mammary cell lines using RNA interference and specific chemical inhibitors.
3. To investigate progestin-induced growth hormone secretion in canine mammary gland. The tissue generated will also form the basis for further gene expression studies.
4. To investigate the involvement of canonical Wnt signaling in progestin-induced canine mammary hyperplasia and spontaneous tumors.
5. To identify overall molecular alterations induced by progestins in canine mammary gland and to identify deregulated cellular process in advanced spontaneous canine mammary tumors.

Chapter 2

General introduction

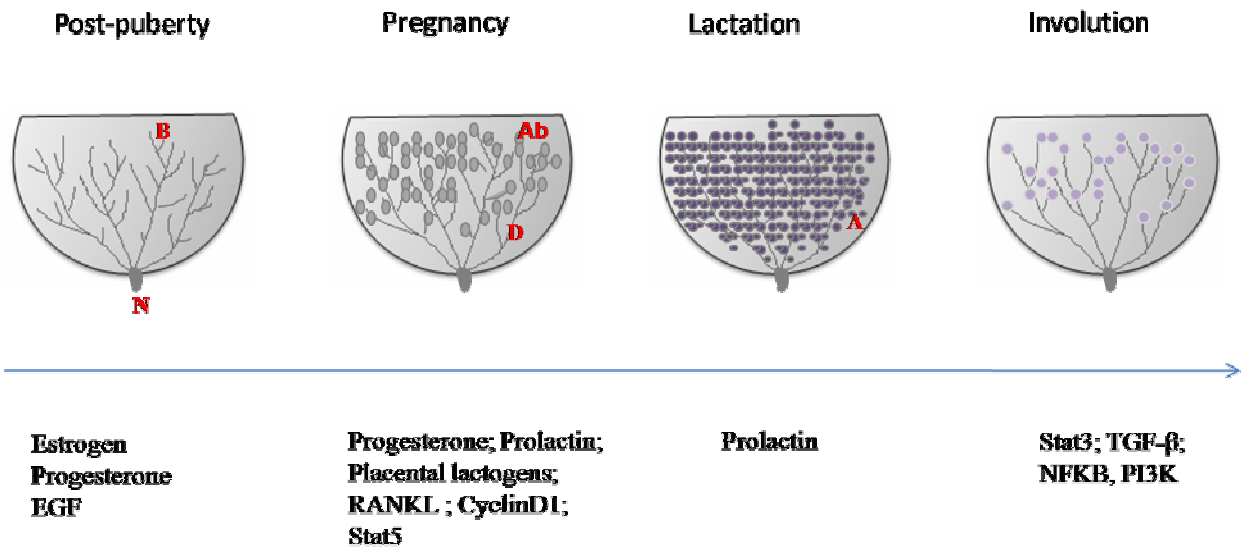
Spontaneous mammary tumors are the most frequently diagnosed malignancy in female dogs. As the majority of mammary cancers in human as well as in the dog are of ductal origin it is important to understand the process of mammary ductal tree development. The key biological processes and/or factors that govern the normal physiological development of the mammary gland may also play a key role in mammary tumor initiation and progression.

Mammary gland development

Our current understanding of the developmental biology of the mammary gland is mainly derived from studies using experiments in rodents. The mammary gland is a branched organ that develops almost completely after birth. Until puberty the gland develops isometrically with the body growth but upon the onset of puberty the mammary gland begins to grow allometrically under the influence of hormones and growth factors. This process involves growth, proliferation, migration, branching and invasion (reviewed in [1]). Pubertal mammary gland development is driven by outgrowth of the highly proliferative terminal end buds (TEB). TEBs invade through the surrounding fat pad to form ducts until they reach the outer limits of the fat pad at which point they regress [2]. TEBs bifurcate to form primary and secondary branches laterally from trailing ducts to form a ductal tree structure [3]. Further, tertiary branches arise from the ductal tree which gives rise to secretory lobuloalveolar structures filling the entire mammary fat pad.

Hormones secreted by the hypothalamus-pituitary-gonadal axis tightly regulate the pubertal mammary gland development. Key steps and growth factors involved in adolescent mammary gland development are highlighted (**Fig 1**.). Ovarian estrogen is the primary hormone that regulates the ductal outgrowth in mammary gland. Estrogen signals through its receptor subtypes ERα and ERβ. However knockout mouse models have revealed that ERα alone is sufficient for ductal outgrowth in mammary gland. ERβ is believed to negatively modulate the actions of ERα [4]. Pituitary growth hormone (GH) is another principal hormone responsible for TEB formation and ductal morphogenesis in mice [5]. GH is known to deliver its effect on mammary ductal morphogenesis through its locally expressed membrane receptor (GHR). Tertiary branching and lobuloalveolar development of the mammary gland is mainly regulated by progesterone [6]. Progesterone is primary secreted by the corpus luteum and binds to the nuclear progesterone receptor that has two receptor isoforms PR-A and PR-B [7-9]. Evidences of

Figure 1. *Schematic diagram depicting important adolescent developmental stages of mammary gland and important growth factors involved in respective stages. (A) functional lobuloalveolar structure; (Ab) alveolar buds; (B) side branches; (D) duct; (N) Nipple. NFκB-nuclear factor of kappa light polypeptide gene enhancer B-cells; RANKL- receptor activator of NFκB; Stat5/3-signal transducer and activator of transcription 5/3; PI3K-phosphatidylinositol 3-kinase [10, 11]*



selective PR-A and PR-B knockout mice suggest that PR-B alone is sufficient for lobuloalveolar development [12]. Progesterone is also known to contribute to the ductal outgrowth by stimulating insulin-like growth factor I (IGF-I) activity [13]. At last the pituitary hormone prolactin, via its membrane receptor is also known to regulate lobuloalveolar development [14].

The nuclear steroid hormone receptors ER and PR utilize a common mechanism of action; they bind to hormone response elements in the promoter region of their target genes and regulate transcription. Specific co-activators and co-repressors, which function in synergy with hormone receptor, allow tissue and stage specific hormone response [15]. Primary mammogens such as estrogen, GH and progesterone are known to regulate ductal/epithelial cell proliferation in a paracrine manner. The steroid hormone receptor expressing cells are distinct and often adjacent to proliferating cells whereby locally induced growth factors mediate the effects of the major mammary hormones [16, 17]. Epidermal growth factor (EGF) and IGF-I are known to be

important local effectors of estrogen and GH, respectively [18, 19]. Similarly, based on studies using mice, Wnt4 is proposed to be a paracrine mediator of progesterone in tertiary branching or alveolar development [20]. Many of these locally induced growth factors are derived from the stromal compartment indicating a strong growth promoting effect of mammary stroma. Growth promoting effects of these stromal growth factors are also counteracted by locally produced growth inhibition factors secreted by ductal epithelium. Transforming growth factor-β (TGF- β) is one of such known inhibitors of ductal elongation and lateral branching [21].

As the TEB moves forward though the fat pad, a drastic extra cellular matrix (ECM) remodeling takes place around the end buds. ECM-active growth factors and matrix modifying enzymes and enzyme inhibitors are important mediators of ECM remodeling. TGF-β is the primary growth factor responsible for inducing matrix re-modeling by inhibiting the matrix degrading proteases and inducing matrix proteins [22]. Matrix metalloproteinases (MMP) are by far the best studied ECM-remodeling enzymes. Among them MMP2 and MMP3 are reported to facilitate ductal elongation by degrading ECM proteins. In addition MMP2 is also known to support cell survival in the end bud [23].

Canine estrous cycle and hormonal milieu

During the estrus cycle and during pregnancy and lactation the mammary gland undergoes distinct phases of tissue remodeling due to hormonal stimulation. After puberty female mammals prepare at regular intervals for possible pregnancy. Females of all species experience a rise in estrogen level as ovarian follicles mature and a rise in progesterone level after ovulation occurs and the corpus luteum develops (luteal phase). These cyclic events which comprise estrous cycle and cycle time are highly variable between species, ranging from a few days in rodents to several months in dogs. Especially dogs have an estrous cycle distinct from other mammals. Dogs reach puberty between 6-18 months and have only one or two estrous cycles per year followed by a prolonged luteal phase (63 days) coinciding with high concentrations of progesterone (similar to pregnancy) [24]. Thus, dogs that are not pregnant following each estrus cycle are still exposed to high concentrations of progesterone without subsequent lactation. This prolonged exposure to progesterone and subsequent absence of lactation is believed to be the key factor contributing to the high prevalence of mammary tumors in non-spayed dogs (reviewed in [25]).

Important hormones and growth factors

Ovarian hormones play a key role in the pathogenesis of mammary cancer [26]. Already in the 1940s estrogens and progestins have been associated with the development of mammary tumors in rodents [27]. However, a much higher impact came from the appearance of mammary tumors in beagle dogs used for toxicity studies of progestins [28]. In 1980 it became apparent that progestin administration to dogs also induced high plasma growth hormone (GH) concentrations [29] that declined after cessation of progestin administration [30]. Not only exogenous progestins but also endogenous progesterone may induce increased plasma concentrations of GH, i.e. in the luteal phase of the estrus cycle [31].

Progesterone

As discussed earlier, progesterone is essential for the development of the mammary gland. But, it may also enhance the risk of mammary tumor formation. Ovariectomy, even when performed at advanced age, protects against mammary tumor formation in the dogs [32]. Binding of progesterone to the PR causes association of the PR dimer with specific DNA sequences, progesterone response elements (PRE), in the promoter regions of target genes. In many cell types the PR-B form acts as a stronger transactivator in comparison to the PR-A form. In the presence of 8-Br-cAMP the PR-B form may respond to the synthetic anti-progestin RU 486 as an agonist [33]. The PR-A form does not show this antagonist/agonist switch activity and seems to be a strong repressor of PR-B. Estrogens, which are known to stimulate the expression of PR, have little or no effect on PR-B concentrations, and act predominantly on the PR-A variant [34]. Transplants of wild-type mammary epithelium into the mammary tissue of PR-/- mice revealed that chimeric epithelium went through complete alveolar development, including the PR-/- cells. This indicates that progesterone stimulates local production of most probably growth factors and that expression of PR is not required in all epithelial cells for normal alveolar development [35]. However, little is known about the downstream effectors of progesterone in mammary gland. A recent study identified involvement of Wnt 4**,** a ligand of canonical Wnt signaling in progesterone-dependent murine mammary gland branching [20]. Studies using PR isoform selective knockout mice have also shown that the receptor activator of nuclear factor kappa B ligand (RANKL) acts as a paracrine mediator of progesterone dependent alveologenesis [36].

These studies indicated a possible involvement of multiple paracrine effectors of progesterone in mammary gland.

Growth hormone (GH)

The expression of pituitary GH is regulated by a variety of transcription factors [37]. In adult dogs both exogenous progestins, given for estrus prevention, as well as endogenous progesterone may evoke a syndrome of GH excess resulting in acromegalic features and insulin resistance [31, 38, 39]. This excessive GH production can be inhibited by antiprogestins, such as RU-486 [40], but is insensitive to stimulation with GHRH or inhibition with somatostatin [41], indicating autonomous GH production. Measurements of GH concentrations in tissue homogenates revealed that progestin-induced GH production in the dog originated from the mammary gland [42]. This was further substantiated by the demonstration of an arterio-venous gradient over the mammary gland and the fast decrease of plasma GH concentrations after complete mammectomy [42]. RT-PCR analysis revealed expression of the gene encoding GH in normal mammary tissue and benign and malignant mammary tumors. GH gene expression was also documented for feline and human mammary tissue [43, 44]

This mammary-derived canine GH appeared to be identical to pituitary GH, however, mammary GH expression occurs in the absence of the transcription factor Pit-1, which is a prerequisite factor for pituitary GH production [45]. Progestin treatment of GH-deficient German shepherd dogs, due to a pituitary anomaly and associated with dwarfism, resulted in improvement of the physical changes and gradually rising plasma IGF-I concentrations [46]. These effects exerted by mammary GH production, further support the difference in the regulation of GH expression between pituitary and mammary tissue. The involvement of the PR in mammary GH expression was confirmed by the immunohistochemical demonstration of the PR in all benign GH-producing mammary cells [47]. However, after malignant transformation mammary GH expression may also occur in PR negative tumors [43, 48]. A recent study has demonstrated that forced expression of human GH is sufficient for oncogenic transformation of immortalized human mammary epithelial cells [49].

The GH receptor (GHR) is a single transmembrane protein which is presented at the cell membrane as a homodimer. After binding of GH, a signal transduction process is stimulated by

activating members of the JAK/STAT pathway in the target cell [50]. In the canine mammary gland a widespread immunohistochemical staining for GHR has been found in normal and tumorous (myo) epithelial cells [51].

Wnt proteins

Wnts are secreted glycoproteins which bind to the extra cellular cysteine-rich domain (CRD) of the frizzled (Fz) family of seven transmembrane receptors and activate the downstream signaling cascade [52]. Intracellular signaling activated by Wnt proteins is thought to be of two main categories: canonical and non-canonical. The canonical Wnt signaling pathway, which involves β-catenin as a key mediator is by far the best characterized of these pathways [53]. Canonical Wnt signaling is triggered when Wnt ligands bind to the Fz receptors and LDL receptor related proteins (LRP5 / LRP6). This mechanism has been the subject of many reviews and is only briefly summarized here [53, 54]. Activated Wnt–receptor complexes act via the cytoplasmic protein Dishevelled to inhibit the activity of a multiprotein β-catenin destruction complex that includes the adenomatous polyposis coli (APC) protein, glycogen synthase kinase 3-beta (GSK3β) and Axin. The function of this complex is to phosphorylate β-catenin and target it for ubiquitination. Once the β-catenin destruction complex is inhibited, β-catenin stabilizes in the cytoplasm and subsequently translocates to nucleus. In the nucleus β-catenin forms a complex with the T-cell-specific transcription factor (TCF) / lymphoid enhancer binding factor 1(LEF1) family of proteins and regulates target gene transcription. Important Wnt target genes include Cyclin D1, cMyc, Stromelysin-1, Survivin, Axin-2 and Cox2 [55].

In the mammary gland, Wnt signals are implicated in the early development, ductal branching and alveolar morphogenesis that occur during pregnancy. Mammary buds fail to form in mice with a disrupted LEF1 locus. LEF1 is normally expressed in the epithelial cells of the mammary bud [56, 57]. In addition, Wnt ligands are also involved in the initiation of early development of mammary buds [58]. Notable increase in the expression of certain Wnt mRNAs in mouse mammary glands was evidenced during pregnancy [59, 60]. These observations suggested that endogenous Wnt proteins may be the local mediators of hormone regulated mammary morphogenesis. In support of this model, Wnt4 was found to be the mediator of progesterone function in mammary lobuloalveolar development [20].

Hyperactivation of the canonical Wnt signaling due to mutations in its key components such as β-catenin, APC and Axin is frequently observed in various human cancers [61-63]. In breast cancer, however, evidences of comparable mutations are lacking. Nevertheless there is strong evidence, based on immunohistochemistry, that more than 50% of breast carcinomas display stabilized β-catenin, an indicator of active canonical Wnt signaling [64, 65]. Overexpression of Wnt proteins has been reported in human breast tumors [66, 67]. Similar to human breast carcinomas, recent studies in canine mammary carcinomas have also indicated increase stabilization of β-catenin [68-71].

Canine mammary tumors

Mammary tumors are the most common type of tumors in non-spayed female dogs. Mammary gland tumors are much more common in countries where ovariohysterectomy is not routinely performed. Recent data from The Norwegian Canine Cancer Register reported a crude incidence of malignant mammary gland tumors of 53.3% in female dogs of any breed [72]. This number appears high but may be influenced by the common use of progestins to prevent estrus in this particular population. The duration of exposure to ovarian hormones early in life determines the overall mammary cancer risk. The risk of developing mammary gland tumors increases from 0.5% to 8%, and to 26%, depending on whether the ovariohysterectomy is performed before the first, second, or after many estrus periods, respectively [73].

In addition to the ovarian hormonal risk for developing mammary gland tumors, there are also other factors, such as genetic predisposition and diet, which have been shown to contribute to tumor development. Certain breeds are known to have an increased risk of developing mammary gland tumors suggesting a genetic predisposition [74, 75]. The tumor suppression gene p53 is the most frequently mutated gene in human tumors, and incidence rate of p53 mutations in canine mammary tumors ranging from 15% to 63% of cases have also been reported [76]. Few selected cases of canine mammary tumors harboring germline mutations in BRCA1 and BRCA2 have been reported [77]. Obesity and a high-fat diet have also been linked to an increased incidence of mammary tumors in dogs similar to that observed in women [78, 79].

Nearly 40-50% of all histologically diagnosed mammary tumors are malignant and the rest being benign tumors [74]**.** Most mammary gland tumors are of epithelial origin (ductal origin)

[80]. **C**ommonly diagnosed benign tumors are adenoma (simple and complex) and benign mixed tumors in the same order. Most frequently occurring malignant tumors are adenocarninomas (tubular /ductal), solid carcinomas and anaplastic carcinomas in that order. Adenocarinoma is believed to be the most differentiated tumor with better prognosis compared to less differentiated solid and anaplastic carcinomas with poor prognosis [80]. Although mammary sarcomas which consist of fibrosarcomas and osteoscarcomas are not common in female dogs, they are known for their poor prognosis [81].

All malignant mammary gland tumors have the potential to metastasize. Important prognostic factors which are used to determine the metastatic potential canine tumors are tumor type, tumor size, histologic staging or invasiveness, and several clinical prognostic factors including immunoreactivity of Ki67 and PCNA [74, 82]. Dogs with malignant mammary gland tumors are known to have a significantly shorter survival time than dogs with benign tumors. The overall 2-year survival has been reported to be between 25% and 40% with a mean survival time ranging from 4 to 17 months, but the survival is influenced by multiple factors, and it can vary significantly depending on histological type and differentiation, stage of disease, and type of treatment [83]. About 50-60% of all malignant tumors contain either progesterone and/or estrogen receptors. ER positive tumors were more differentiated and less aggressive compared to ER negative tumors [73, 84]. In human breast cancer, expression of c-erbB-2 (HER2/neu) has been associated with a more aggressive phenotype. One study identified 35% of malignant canine mammary tumors showing immunoreactivity to c-erbB2 which was found to be positively associated with histological grade and mitotic count [85].

Currently, surgery is the only commonly practiced treatment for dogs with mammary tumors, and there are no established adjuvant chemotherapy or hormonal therapies as practiced in human medicine [86]. There is, however, limited information regarding efficacy of adjuvant chemotherapy in dogs with high-risk mammary gland tumors, and only a single study reporting the efficacy of chemotherapy in dogs with gross metastases [87]. Doxorubicin was found to be effective in dogs with pulmonary metastasis, and a preliminary report showed improved survival in dogs with high-risk mammary gland tumors receiving adjuvant doxorubicin compared with

dogs treated with surgery alone [88]. There are no randomized studies on the effect of adjuvant chemotherapy in dogs with high-risk mammary gland tumors.

Consistently the high mortality rate of female dogs after surgical resection of a malignant mammary tumor demonstrates that current treatment regimes are not very effective [73, 74, 89]. This highlights the need for more prospective studies to evaluate the efficacy of chemotherapies in dogs with high risk mammary gland tumors. Better understanding of the molecular alterations in canine mammary tumors may help to identify new markers and therapeutic targets. A great part of molecular understanding of human breast carcinomas is based on *in vivo* and *in vitro* studies done on breast cancer cell lines apart from rodent studies. Advent of microarray has greatly contributed to the understanding of the molecular etiology of human breast cancer. Many microarray studies have successfully characterized human breast carcinomas and cell lines [90-92]. Many aspects of canine mammary tumors are similar to their human counterparts. Even the local mammary expression of GH, which is thought to be a unique feature of progestin treatment in the dog, has also been demonstrated in humans. The role of progestins, however, has not yet been demonstrated in humans. The lessons that can be learned from comparative oncology warrants the need for further molecular understanding of the disease in dogs.

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Chapter 3

cDNA microarray profiles of canine mammary tumour cell lines reveal deregulated pathways pertaining to their phenotype

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Abstract

Mammary cancer is the most common type of cancer in female dogs with a life-time risk of over 24% when dogs are not spayed. The elucidation of the complete canine genome opens new areas for development of cancer therapies. These should be tested first by *in vitro* models such as cell lines, however to date, no canine mammary cell lines have been characterized by expression profiling. In this study, canine mammary tumour cell lines with histologically distinct primary tumours of origin were characterized using a newly developed canine cDNA microarray. Comparisons of gene expression profiles showed enrichment for distinct biological pathways and were related to biological properties of the cell lines such as growth rate and *in vitro* tumourigenicity. Additionally, gene expression profiles of cell lines also showed correspondence to their tumour of origin. Major differences were found in Wnt, cell cycle, cytokine/Rho-GTPase, alternative complement and integrin signalling pathways. As these pathways show an overlap at the molecular level with those found in human breast cancer, the expression profiling of spontaneous canine mammary cancer may also function as a biological sieve to identify conserved gene expression or pathway profiles of evolutionary significance that are involved in tumourigenesis. These results are the basis for further characterization of canine mammary carcinomas and development of new therapies directed towards specific pathways. In addition these cell lines can be used to further investigate identified deregulated pathways and characterize until now unannotated genes.

Introduction

Mammary cancer is the most common type of cancer in female dogs where its incidence and mortality rates are at least twice as high as in humans [1]. About 50% of canine mammary tumours are classified as malignant, and most of the treatment strategies (such as combinations of chemotherapies, radiation and immunotherapy) are not very effective at preventing recurrence [1]. Early ovariectomy is reported to reduce the life time mammary cancer risk from 24% to 0.05% and even when performed on older dogs, partially protects them against mammary tumour formation [2, 3] in agreement with the hormone dependence of the disease. In dogs, progestins play a major role in the incidence of mammary tumours, at least in part through stimulation of mammary growth hormone expression [4-6].

Breast cancer is also the most common cancer in women comprising 23% of all female cancers [7]. The etiology of breast cancer is very complex and not very well understood. In addition to diverse genetic, dietary, environmental and carcinogenic entities leading to tumourigenesis, breast cancer has long been recognized as a hormone dependent malignancy [8]. The role of ovarian hormones, predominantly estrogens and to a lesser extent progestins in mammary gland development, tumourigenesis and tumour progression have been very well documented by many studies (reviewed [8]).

Although the advent of DNA microarray technology has led to numerous gene expression profiling studies of (human) breast tumours and cell lines which have been reported recently [9-11], in the dog, no gene expression profiling studies have yet been documented. There are very few well characterized (at the molecular level) canine mammary tumour cell lines (CMTs) available for mammary cancer research in dogs but no studies are reported which identify main signaling pathways (in CMTs) or make cross species comparisons of pathway profiles to identify molecular similarities of the disease across species.

In this study, as a first step we developed a dog specific cDNA microarray containing 20,160 independent genes and studied the gene expression signatures of three CMTs originating from a primary atypical benign mixed tumour (CMT-U229), a primary mammary osteosarcoma (CMT-U335) and a primary mammary anaplastic carcinoma (P114). Several differentially

expressed gene clusters were identified, related to various signalling pathways and were compared to gene expression profiles in human breast cancer. These results are the basis for further characterization of canine mammary carcinomas.

Materials and Methods

Cell lines and cell culture

The cell line CMT-U335 was isolated from a canine mammary osteosarcoma, CMT-U229 from a canine mammary atypical benign mixed tumour and P114 from a highly malignant canine anaplastic carcinoma. Isolation and characterization of these three cell lines were reported previously [12, 13]. Cell lines were grown in DMEM-F12 (Invitrogen, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Harlan Sera-Lab, UK) at 37^0C in a humidified atmosphere of 5% CO_2. All experiments were conducted at early passages of cell lines (3-22 passages).

Growth rate experiments were performed in triplicates. For this, 5×10^4 cells were seeded into each well of 6-well plates (Becton Dickenson, Germany). Cells were counted manually after being treated with trypsin every 24 hours for 4 days. The experiment was repeated 5-6 times and the average doubling time of each cell line was estimated. These results were validated by estimating the doubling time using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay as described previously with minor modification to the original protocol [14]. In brief, cell lines were seeded in 96-well plates (Becton Dickenson, The Netherlands) at a density of 1500 - 3000 cells / well. After 24, 48 and 72 hours, the cells were incubated with MTT (0.45 mg / ml of final concentration) for 1.5 hours. After 1.5 hours the cell culture medium with MTT was removed, cells were lysed with acidic Isopropanol (100 µl / well) and the absorbance of the dissolved formazan was measured at 595 nM with 650 nM as reference wavelength using a microplate reader (BioRad, Model 3550). Cell number for every time point was deduced from absorbance using the standard curve (serial dilution of cells) for each cell line.

Growth characteristics of the cell lines were documented by growing cell lines in reconstituted matrigel matrix (Becton Dickenson, Germany). For this, cell lines were seeded at 5×10^4 cells per well in matrigel coated 12-well plates (Becton Dickenson, Germany) and the

morphology of cells was assessed under IMT-2 phase contrast microscope fitted with E-330 digital camera (Olympus, The Netherlands) after one week. Experiments were repeated 5-6 times.

Invasion assay

Invasion assays were performed as described earlier [15] using Transwell chambers (Corning, The Netherlands) with minor alterations to the original protocol. The 8 μm pore membranes of upper chambers were coated with 75 μg (50 μl) of Matrigel (Becton Dickenson, Germany) and placed into a well containing DMEM-F12 supplemented with 10% FBS. About $1x10^5$ cells in media containing 1% FBS were seeded into the upper chamber of transwells. After 20 hours of incubation, cells were mechanically removed from the upper compartment of the transwell chamber by using cotton swabs. The number of cells which have passed through the matrigel and filter membrane of transwells into the lower chamber was estimated using the DNA based sensitive Cyquant cell proliferation kit (Invitrogen, The Netherlands). Standard curves were plotted by serial dilution of cell lines and their corresponding fluorescence signal. Total number of invaded cells was deduced for each cell line from their respective standard curves. Invaded cells were also visually inspected under the phase contrast microscope. All assays were repeated 3-4 times.

Colony formation in soft agar

The anchorage-independent growth ability of cell lines was determined by colony formation in soft agar as described earlier [16] with minor modifications. Single-cell suspension of each cell line was prepared from monolayer culture by briefly treating them with trypsin. Cells (3×10^4 cells) suspended in 1.5 ml of DMEM-F12 containing 10% FBS and 0.5% low melting point agarose (Promega, Netherlands) were seeded into each well of 6-well plates pre-coated with a 1.5 ml layer of solidified DMEM-F12/10% FBS / 1% agarose. Cell culture medium was refreshed every 3-4 days. Two weeks later, colonies were photographed under 10 -12 random focus areas using the IMT-2 phase contrast microscope fitted with E-330 digital camera (Olympus, The Netherlands) at 10x magnification. The experiments were performed in triplicate and repeated 4 times. Individual colonies were scored for each focus area and the average number of colonies for each area was calculated.

Canine specific microarray production

A canine specific collection of 20,160 non-redundant clones of 3'-UTR cDNA fragments inserted in pBluescript II KS and cloned into E Coli-DH10B were purchased from Lion Bioscience GmbH. Individual clones were grown overnight in LB medium supplemented with 50 µg/ml ampicillin and heated at 95^0C for 10 min. Four µl of bacterial culture was subsequently used for PCR amplification of the clone inserts using plasmid specific sense 5'-AGCGTGGTCGCGGCCGAGGT and antisense 5'-TCGAGCGGCCGCCCGGGCAGGT primers and 35 cycles of denaturation (94^0C, 30 sec), annealing (66^0C, 30 sec) and extension (72^0C, 1 min) using the standard PCR protocols. PCRs with 100 µl reaction volumes were performed in triplicate for each clone in order to obtain enough PCR products for microarray production. Each PCR product was checked for contaminations using gel electrophoresis, and flagged if no or more than one PCR product was detected. PCR products were purified using 96-well MultiScreen plates with FB filters according to the manufacturer's protocol (Millipore, The Netherlands). After measuring the concentration, PCR products were air dried, reconstituted to 150 ng/µl of final concentration in print buffer (150 mM phosphate buffer pH 8.5) and 10 µl of each product was transferred to 384-well plates. PCR products that did not reach the desired concentration were flagged. PCR products were spotted on UltraGAPS slides (Corning, The Netherlands) using the MicroGRID II arrayer (Genomic Solutions, UK), air dried and immobilized by UV cross linking using a Stratalinker2400 (Stratagene, CA) for 10 min at 2400 mJ. Microarrays were stored at room temperature (RT) in desiccators until needed. The microarray platform has been deposited in the public database [GEO: GPL5117].

RNA isolation and cRNA synthesis

Labelled cRNA was prepared according to a previously published protocol [17, 18]. RNA isolation and purification was performed with the RNAeasy Mini Kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Total RNA was isolated from cells which were grown to near confluence in 75 cm^2 Cellstar tissue culture flasks (Greiner Bio-One GmbH, The Netherlands). Total RNA from 3-4 independent passages of each cell line was used for cRNA synthesis. About 2 µg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, The Netherlands) and T7 promoter coupled poly T primers (5'

GGCCAGTGAATTGTAATACGACTCACCTATAGGGAGG CGGT). After second strand synthesis, cDNA products were purified using QIAquick PCR Purification Kit (Qiagen, The Netherlands), followed by transcription using the T7 Megascript kit (Ambion, The Netherlands) and a UTP to 5-(3-aminoallyl)-UTP ratio of 1/3.5. The RNeasy mini kit (Qiagen) was used to purify the resulting cRNA. The quality of the total RNA and the amplified cRNA was analyzed using a bioanalyzer (Agilent technologies, The Netherlands). Three µg cRNA that met the quality criteria was coupled to Cy3 and Cy5 fluorophores (GE Healthcare, Belgium). Labeled cRNA was purified using ChromaSpin-30 columns (Clontech-Takara Bio, France), and dye incorporation efficiency was measured using a ND-1000 spectrophotometer (Isogen, The Netherlands). Equal quantities of labeled cRNA samples with specific activity of 2-5% dye-labelled nucleosides were used for microarray hybridization.

Microarray hybridization and scanning

Microarray slides were washed for 1 min in 2x SSC containing 0.05% SDS at RT, incubated in 0.25% sodium borohydride and 0.25% SDS in 2x SSC for 30 min at 42^0C and then rinsed 5 times in double distilled water at RT. Slides were incubated for 45 min at 42^0C in pre-warmed pre-hybridization buffer (2X) consisting of 10xSSC, 25% formamide, 0.1% SDS and 1% bovine serum albumin (BSA). Slides were washed 5 times in water followed by 5 times dip in isopropanol at RT and air dried. To 40 µl mixture containing 2.5 µg of each Cy3 and Cy5 labelled RNA, 40 µl hybridization buffer consisting of 50% formamide, 10xSSC, 0.2% SDS and 200µg/ml sheared herring sperm DNA was added. The mixture was heated at 95°C for 5 min, centrifuged for 2 min at 12,000xg and hybridized to the slides at 42^0C for 16-18 hours using hybridization chambers (Corning, The Netherlands) in a water bath. After hybridization, slides were washed 4 times: first a low-stringency wash for 4 min at RT in 1xSSC and 0.2% SDS, next a high-stringency wash in 0.1xSSC and 0.2% SDS for 4 min and finally 2 washes in 0.1xSSC at RT. Slides were blown dry using compressed nitrogen and scanned using an Agilent G2565AA DNA microarray scanner (100% laser power, 30% photomultiplier tube).

Data analysis

Image analysis was carried out using Imagene 5.0 software (Biodiscovery Inc, The Netherlands). Defective spots were flagged and data was normalized based on Lowess print-tip normalization [19] by using the gene spots. Normalized data was subjected to logarithmic (base 2) transformation before further statistical analysis. Differentially expressed genes between cell lines were extracted by performing a two class unpaired t-test using significance analysis of microarray (SAM) software with the mean false discovery rate set at 5% [20]. Gene expression data were

Table 1. Nucleotide sequences of canine specific primers pairs used for quantitative real time-PCR analysis. U = forward primer; L = reverse primer; T = optimum annealing temperature; bp = base pairs.

Gene name	Primer orientation	Primer sequences (5'-3')	T (^{0}C)	Product size (bp)
SFRP1	U	AGCGAGTTTGCATTGAGGAT	60	106
	L	TCTTGATGGGTCCCAACTTC		
CRYAB	U	CCATGCACCTCAATCACATC	58	104
	L	ATGCGTCTGGAGAAGGACAG		
BF	U	AGGGACACGAAACCTGTATG	62	106
	L	ACGCTGACCTTGATTGAGTG		
KTR8	U	CCTTAGGCGGGTCTCTCGTA	63	149
	L	GGGAAGCTGGTGTCTGAGTC		
DDR1	U	CTGGGGTCAGGAGGTGATT	58	135
	L	ACCCGCAGACAGACACTCAT		
CDKN1A	U	ATGAAATGGGGGAAGGGTAG	58	116
	L	AATCTGTCAGGGGCGTATTG		
RGC32	U	CATCTCTGTCGGCTCTGGTAG	58	99
	L	AAGTTCTGGGTCCTTTCATCAT		
TMEFF2	U	TAGTCCAGCCACTGTGCAAC	58	141
	L	CCTCTGCATCACCAGGAAAT		
GP80	U	CCGAGAGGAATGAGATGTGAA	57	111
	L	CACTTGGTGACATGCAGAGC		
DKK3	U	CATCCAGTCCAGTGCTCTCA	58	140
	L	GGGCCAGGATTGTAAGTGAA		
PAK1	U	CCCAAGGTTGACATCTGGTC	58	110
	L	TTGGTGGCAATGAGGTACAA		
RPS19	U	CCTTCCTCAAAAAGTCTGGG	61	95
	L	GTTCTCATCGTAGGGAGCAAG		
HPRT	U	AGCTTGCTGGTGAAAAGGAC	56	100
	L	TTATAGTCAAGGGCATATCC		

clustered and visualized using Genespring version 7 (Agilent technologies, The Netherlands).
SAM generated and annotated differentially expressed genes were subjected to pathway analysis in the framework of gene ontology (GO) and signalling networks using the Panther pathway analysis tool [21]. Major pathways overrepresented by the statistically significant genes (random overlapping p value <0.001) were identified as important signalling pathways. The microarray data files have been deposited in the public database [GEO: GSE7659].

Real time quantitative PCR (RT-PCR)

Synthesis of cDNA was carried out with 1.5 µg total RNA in 60 µl reaction volumes using the iScript™ cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, The Netherlands). Primers (Table 1) were designed using primer select software of DNA star (Madison, WI) according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product. HPRT and RPS19 genes were used as the non-regulated reference genes for normalization of target gene expression [22]. RT-PCR was performed as described previously using Bio-Rad MyIQ detection system (BioRad, The Netherlands) with SYBR green fluorophore. Data analysis was carried out using the pairwise fixed reallocation and randomization test incorporated in the software program REST-XL [23] at 5% level of significance. Estimated fold change for each gene was plotted against the same estimated from the microarray analysis.

Results

Growth characteristics of canine mammary cell lines

Canine mammary cell lines showed a clear difference in growth velocity when grown on plastic cell culture bottles (Fig.1). CMT-U229 showed highest doubling times (18±2 h) followed by P114 (22 ±2 h) and CMT-U335 (30±2 h). When grown in laminin rich matrigel matrix, cell line P114 formed branching structures (Fig. 1). Although, CMT-U229 and CMT-U335 formed colonies in matrigel, they showed no signs of branching morphogenesis. Compared to CMT-U229 and CMT-U335, P114 was observed to be highly tumourigenic with greater invasion ability in the transwell invasion assay and higher anchorage-independent growth ability when grown in soft agar (Fig. 2).

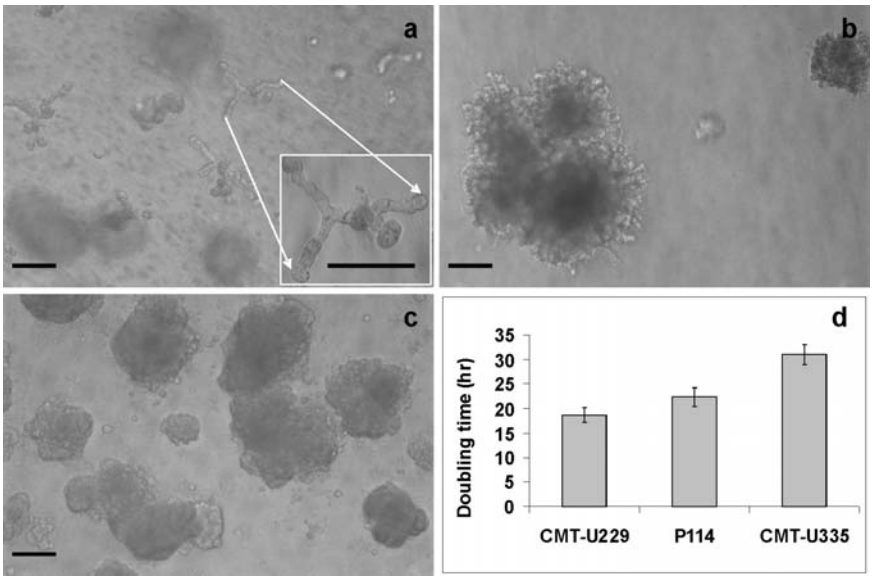

Figure 1. *Growth characteristics of CMTs: Phase contrast micrograph of CMTs grown on matrigel matrix for a week. (— bar = 300µm).* ***a****- P114, insert = 20x magnification;* ***b****- CMT-U335;* ***C****- CMT-U229;* ***c****- doubling time of CMTs when grown on plastic growth surface.*

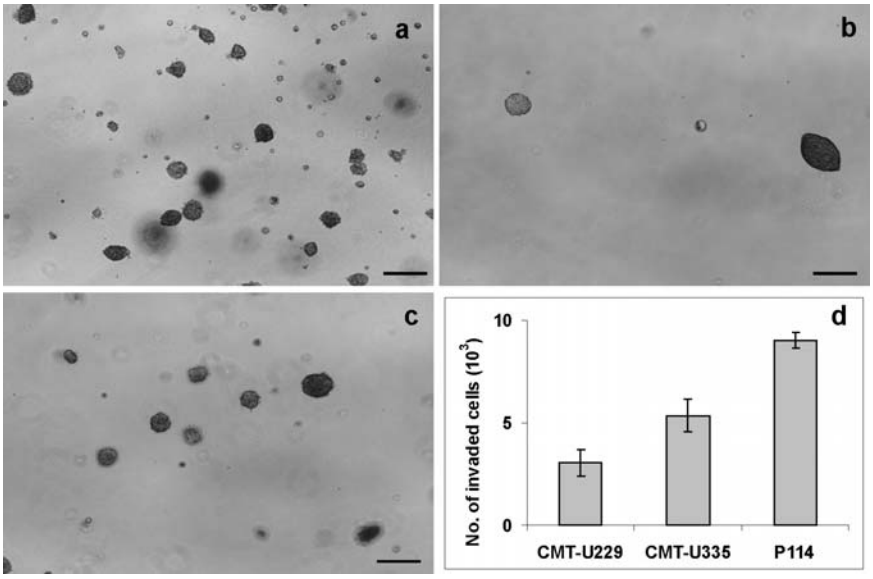

Figure 2. *Tumourigenicity of CMTs: Anchorage independent growth ability of CMT's grown in soft agar over two weeks. Representative phase contrast micrographs are presented (— bar = 300µm).* ***a****-P114;* ***b*** *-CMT-U229;* ***c*** *-CMT-U335.* ***d*** *-Invasiveness of CMTs estimated as number of cells invaded through matrigel coated transwell chambers in 20 hours over a serum gradient.*

Gene expression profiling of canine mammary cell lines

The three canine mammary cell lines we characterized using canine specific cDNA microarray, originated from histologically distinct primary tumours. Statistical analysis of raw data using significance analysis for microarrays (SAM) with a false discovery rate set below 5% generated a clear set of genes differentially expressed between cell lines (Supplementary Table 1). CMT-U229 overexpressed 70 genes against P114 and 179 genes against CMT-U335. The cell line P114 overexpressed 118 genes compared to CMT-U335 and 70 genes compared to CMT-U229. The cell line CMT-U335 overexpressed 165 compared to CMT-U229 and 93 genes compared to

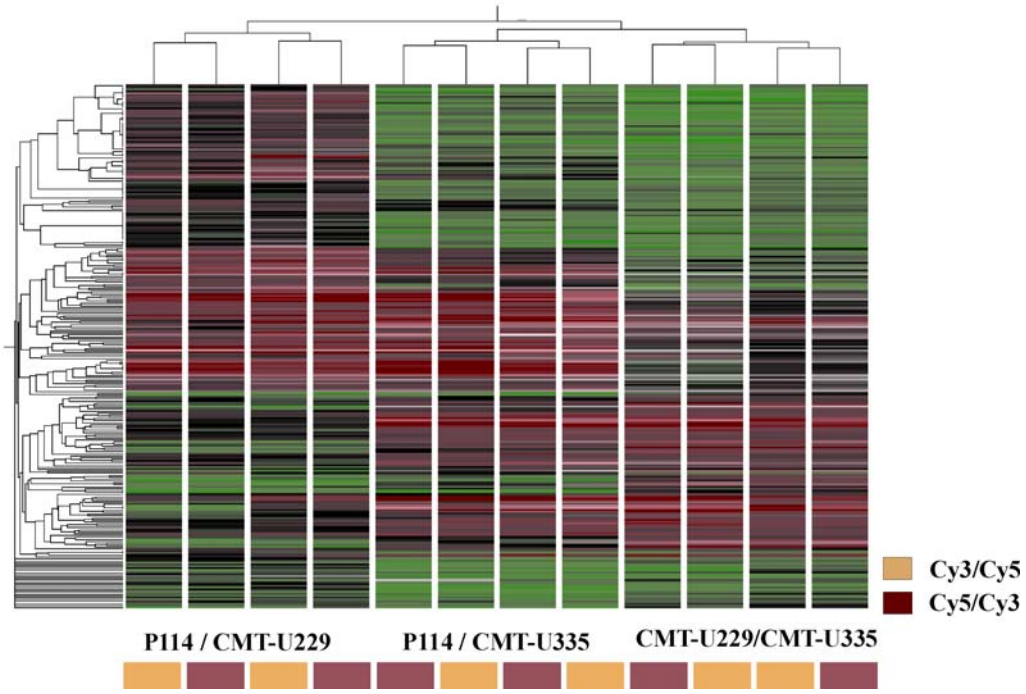

Figure 3. *Dendrogram generated (in Genespring) by hierarchical clustering of all differentially expressed genes in CMTs found by SAM analysis revealed a clear clustering of replicate experiments and their dye swaps.*

P114. The statistically significant gene lists obtained from SAM analysis were further analyzed and visualized using Genespring software. Dendrograms generated by hierarchical clustering of gene expression data revealed a clear clustering of replicate experiments and their dye swaps (Fig 3).

Unique gene lists, which are the lists of genes that are differentially expressed in a particular cell line compared to other two cell lines, were generated. In CMT-U229, 32 unique upregulated and 7 downregulated genes were found. P114 showed 36 upregulated and 15 downregulated genes. CMT-U335 had 53 upregulated and 45 downregulated genes (Supplementary Table 2).

Gene expression data were validated by RT-PCR on differentially expressed candidate genes using cell line total RNA used for microarray analysis as well as RNA derived from different biological replicates of each cell line. A good correlation (R^2=0.753) was found between the fold change values obtained by microarray analysis and RT-PCR quantification for the randomly selected set of candidate genes (Fig. 4).

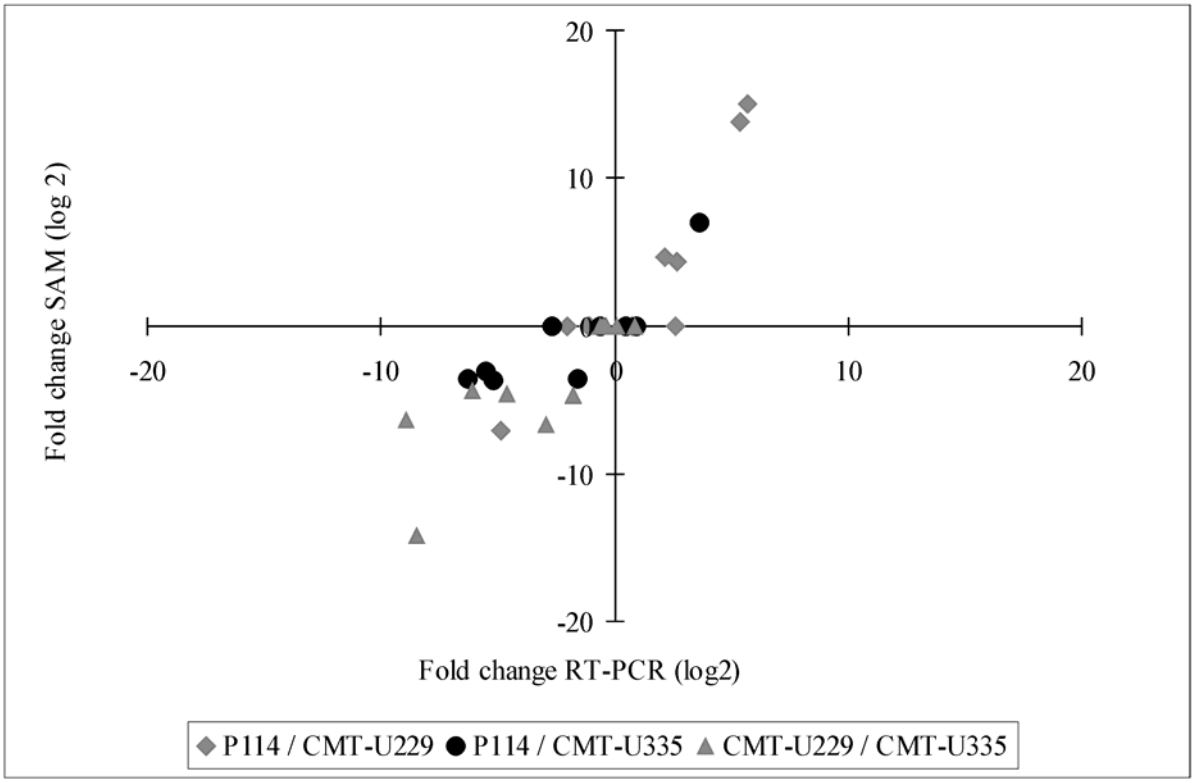

Figure 4. *Correlation between gene expression values of few candidate genes obtained by microarray analysis (SAM) and quantitative real time PCR (log scale).*

Table 2. *Statistically significant differentially expressed genes in CMTs with known functional annotation were sub-grouped under relevant biological pathways. Only important signalling pathways represented by large number of differentially expressed genes are shown: (↓) = gene downregulated against one cell line; (↓↓) = gene downregulated against two cell lines; (↑) = gene upregulated against one cell line; (↑↑) = gene upregulated against two cell lines; (+) = stimulation of the pathway or its effectors; (-) = inhibition of the pathway or its effectors.*

Gene ID	Ref-seq-HS	Gene name	Regulation	Cell lines		
				CMT-U229	*P114*	*CMT-U335*
Wnt-signalling						
DG2-66o13	NM_000944	PPP3CA	+	↑	≈	↓
DG9-288i19	NM_002074	GNB1	+	↓	≈	↑
DG40-163k12	NM_001892	CSNK1A1	+	↑	↑	↓
DG32-147a19	NM_053056	CCND1	+	↑	↑	↓
DG11-72g24	NM_004284	CHD1L	+	≈	↑	↓
DG9-113g15	NM_001257	CDH13	+	↓	↑	↑
DG2-88c13	NM_001904	CTNNB1	+	↓	↑	↓
DG2-7i22	NM_004932	CDH6	+	↓	↓	↑
DG32-213c20	NM_007236	CHP	-	↑	≈	↓
DG2-126g23	NM_001329	CTBP2	-	≈	↑	↓
DG32-129f24	NM_005077	TLE1	-	↓	↑	↑↑
DG2-102p15	NM_013253	DKK3	-	↑	↓	↓↓
DG9-275n20	NM_003012	SFRP1	-	↑	↓↓	↓
DG8-49l18	NM_001954	DDR1	+	↓	↑	↓
DG2-102m21	NM_000582	OPN/SPP1	+	↓	↑	↓
Cell cycle and Alternative complement cascade						
DG32-147a19	NM_053056	CCND1	+	↑	↑	↓
DG33-101c10	NM_004060	CCNG1	+	↑	↑	↓
DG11-192m15	NM_006835	CCNI	+	↑	↓	↑
DG2-41a10	NM_000321	RB1	-	≈	↑	↓
DG2-34n17	NM_000389	CDKN1A	-	↓	↑	↓↓
DG12-1a17	NM_001710	BF	+	↑	↓	↓
DG10-1p13	NM_001710	BF	+	↑	↓	↓
DG2-36l8	NM_014059	RGC32	-	↑	↓	↓↓
DG11-4j12	NM_014059	RGC32	-	↑	↓	↓↓
DG32-152k14	NM_004048	B2M	-	↑	↓	↓
DG2-93n14	NM_001831	CLU	-	↑	↓	↓

Gene ID	Ref-seq-HS	Gene name	Regulation	Cell lines		
				CMT-U229	*P114*	*CMT-U335*
DG2-10i18	NM_014011	SOCS5	-	↑	↓	↓
Integrin-signalling						
DG33-90p14	NM_000090	COL3A1	+	↓	↑	≈
DG8-53o24	NM_001753	CAV1	-	↓	↑	≈
DG2-66a5	NM_005717	ARPC5	+	≈	↓	↑
DG11-126m15	NM_002213	ITGB5	+	↓	≈	↑
DG2-133o11	NM_001315	MAPK14	+	↓	≈	↑
DG33-2h3	NM_003373	VCL	+	↓	≈	↑
DG9-140l22	NM_004370	COL12A1	+	↓	↓	↑
DG40-161p20	NM_000089	COL1A2	+	↓↓	↓	↑
DG2-64i21	NM_001012267	OGN	+	↓↓	≈	↑
DG14-240i5	NM_001845	COL4A1	+	↓↓	↓	↑
DG2-28j3	NM_001010942	RAP1B	+	↓	↑	↑↑
DG42-169k17	NM_002026	FN1	+	≈	↑	↓
DG14-194i10	NM_001848	COL6A1	+	↑	≈	↓
DG33-32n23	NM_000954	LAMB2	+	↑	≈	↓
DG14-5c14	NM_000210	ITGA6	+	↑	≈	↓
DG8-173b12	NM_000213	ITGB4	+	↑	≈	↓
DG2-68i3	NM_002291	LAMB1	+	↓	↑	↓
DG11-131c5	NM_000227	LAMA3	+	↓↓	↑	↓
Cytoskeletal / Rho-GTPase signalling						
DG14-194i10	NM_001848	COL6A1	+	↑	≈	↓
DG11-93m20	NM_138455	CTHRC1	+	↑	≈	↓
DG11-175f19	NM_138455	CTHRC1	+	↑	≈	↓
DG2-24b7	NM_002576	PAK1	+	↑	≈	↓
DG32-41p15	NM_005965	MYLK	+	↑	↓	↓
DG2-105g11	NM_030808	MYH10	+	≈	↓	↑
DG2-28b10	NM_053024	PFN2	+	≈	↓	↑

Pathway analysis

Annotated differentially expressed gene lists of all the three cell lines were analyzed using Panther Pathway analysis tool (Table 2). In CMT-U229, many key cell cycle activating cyclins (CCND1, CCNG1 and CCNI) are overexpressed and cell cycle inhibitor (CDKN1A) is underexpressed. Interestingly, RGC32, a complement-signalling induced gene believed to have a

role in the cell cycle and BF, a gene involved in the assembly of membrane attack complex of complement cascade is also upregulated in this cell line. Genes involved in cytokine/Rho-GTPase signalling (SOCS5, PAK1 and MYLK) and integrins ITGA6 and ITGB4 are also overexpressed in CMT-U229. Cell line P114 had high expression of many activators and direct targets of the Wnt pathway (CSNK1A1, CTNNB1, CCND1, DDR1 and OPN) and lower levels of inhibitors of the Wnt pathway (DKK3, SFRP1). P114 also overexpressed cell cycle inhibitor CDKN1A, laminins (LAMA3 and LAMB1) and fibronectin (FN1). CMT-U335 overexpressed many genes involved in integrin signalling such as collagens (COL12A1, COL1A2 and COL4A1), OGN, RAP1B, VCL, ITGB5 and MAPK14.

To gain additional insight, we compared the pathway profiles with those of the human gene sets [11, 24]. Sorlie *et al* identified five distinct molecular subtypes of human breast carcinomas using the expression pattern of an intrinsic gene set (≈500 genes). Charafe-Jauffret *et al* identified differentially expressed genes (≈1400 genes) between basal, luminal and mesenchymal subtypes of human breast cancer cell lines. Direct comparison of these human gene sets with canine cell line data was impossible because of a lack of representation of these genes on the canine microarray. Instead, the CMTs gene set of all differentially expressed genes, human breast cancer cell line gene set and breast carcinoma intrinsic gene set were subjected to the Panther^{TM} pathway analysis. Even though the majority of the genes in each gene set were not classified under any signalling network, pathway analysis identified a striking similarity in the pathway profiles of these three gene sets. Four pathways (Integrin, Wnt, angiogenesis and cytokine/chemokine signalling) were among the top 5 pathways commonly identified in all three gene sets (Table 3.). Parkinson's disease, Alzheimer's disease and PDGF signalling pathways were differentially represented among three gene sets (Table 3).

Discussion

With the advent of microarray technology, there have been many reports on the molecular characterization of human breast tumours and cancer cell lines. Five different subtypes (luminal-A, luminal-B, basal, ERBB2-amplified and normal-like) of human breast tumours have been identified using an intrinsic gene set as classifier [11]. In a similar study, basal, luminal and

Table 3. *Major (Top 5) deregulated cellular signalling pathways in CMTs, human breast cancer cell lines (BCL) and human breast carcinomas (BC). List of all differentially expressed genes in CMTs, and previously published BCL (Charafe-Jauffret et al 2006) and BC (Sorlie T et al 2003) gene lists were independently compared to a common reference gene list available in panther pathway database for their over representation in cellular signalling pathways: p value = random overlapping p value; No. of genes/% Genes = number/percentage of genes in each gene list.*

Panther major pathways hits (p<0.001)	No. of genes	% Genes	p value
Canine mammary cell line gene list			
Integrin signalling pathway	17	7.70	0.00034
Wnt-signalling pathway	15	7.30	0.00057
Parkinson disease	11	5.30	0.00049
Chemokine and cytokine signalling pathway	8	3.80	0.00031
Angiogenesis	6	2.90	0.00005
Human breast cell line gene set			
Integrin signalling pathway	29	5.20	0.00034
Angiogenesis	27	4.80	0.00005
Wnt-signalling pathway	23	4.10	0.00057
Chemokine and cytokine signalling pathway	23	4.10	0.00031
PDGF signalling pathway	18	3.20	0.00047
Human breast cancer gene set			
Wnt-signalling pathway	15	6.50	0.00057
Alzheimer disease-presenilin pathway	12	5.20	0.00004
Angiogenesis	10	4.30	0.00005
Integrin signalling pathway	8	3.50	0.00034
Chemokine and cytokine signalling pathway	8	3.50	0.00031

mesenchymal subtypes of human breast cancer cell lines were identified [24]. To the best of our knowledge this study is the first microarray study to use dog mammary tumour cell lines. In this study we characterized three well established CMTs originating from totally different types of primary tumours with distinct biological behaviours. Two of these three cell lines carry missense point mutations in the DNA binding domain of tumour suppressor p53 (CMT-U335 and CMT-U229), whereas P114 carries a wild type p53 [13]. Genomic instability (amplification/deletion) is a frequently observed phenomenon in tumours and tumour cell lines which may influence their

gene expression profile. However, we did not observe a significant number of differentially expressed genes being localized to a single chromosomal area in CMTs indicating low chances of karyotypic instability induced bias in the CMT gene expression profiles. A comparative genomic hybridization (CGH) analysis using a recently developed canine CGH array [25] in combination with the gene expression profile may have, however, a synergistic effect on the understanding of the development of deregulated pathways in canine mammary tumours.

Cell line CMT-U229

The CMT-U229 cell line originated from a canine atypical benign mixed tumour. Although rare in human breast cancer, they are common in dogs. Benign mixed tumours usually contain epithelial, mesenchymal and myoepithelial cell types. Although benign, they are known to carry clonal karyotypic abnormalities probably indicating potential malignancy [26]. In a proliferation assay CMT-U229 showed the fastest growth rate compared to CMT-U335 and P114. This high growth rate and previously reported high immunoreactivity to epithelial markers and low reactivity to mesenchymal marker vimentin [12] indicate that this cell line might have evolved from a small population of malignant epithelial cells of the parent tumour. Alternatively it might have acquired the malignancy *in vitro* which is supported by its ability to form anaplastic tumours when inoculated into nude mice [12].

Pathway analysis of the gene expression data indicated an enrichment of candidate genes involved in the immune system-mediated signalling (B2M, CLU, BF, RGC32 and SOCS5) in CMT-U229 (Table 2). The major histocompatibility complex-1 (MHC-1) is of central importance in regulating immune response against tumour cells. Increased expression of beta-2-microglobulin (B2M), a component of MHC-1, by tumour cells has significance with regard to escape from immune-surveillance and cell survival [27, 28]. Clusterin (CLU) is an important glycoprotein which biologically binds to MHC-1 complex rendering it inactive and making tumour cells resistant to cytolysis mediated by MHC-1 [29]. CLU is also reported to inhibit the BAX family of genes mediated apoptosis making tumour cells resistant to apoptosis [30] and it is found to be overexpressed in many solid tumours including human breast carcinomas [31]. Factor B (BF) is a component of alternative complement pathway of immune signalling and its exact role in tumourigenesis is yet to be investigated. Response gene to complement 32 (RGC-32) is known to

be involved in complement pathway mediated cell cycle progression and is reported to be overexpressed in many solid tumours [32, 33]. Suppressor of cytokine signalling (SOCS5) is found be overexpressed in many solid tumours and cell lines and is attributed to their resistance to pro inflammatory cytokines [34]. Over expression of the above discussed genes in CMT-U229 may partially contribute to its high growth rate by making it resistant to apoptosis or by stimulating the cell cycle.

Cell cycle aberrations are the most common phenomena observed in tumourigenesis. Cell cycle activating cyclins (CCND1, CCNG1 and CCNI) and cell cycle inhibitor CDKN1A ($p21^{waf\ cip}$) are upregulated in CMT-U229 indicating deregulated cell cycle which could be another cause for its high growth rate. Apart from cell cycle genes, oncogenic integrins, namely α-6 and β-4 integrins (ITGA6 and ITGB4) are overexpressed in CMT-U229. The role of ITGB4 and ITGA6 is also highlighted in increased cell survival, anchorage-independence and invasion potential of many types of solid tumours, including breast tumours [35]. Overexpression of these integrins in CMT-U229 is in contrast to its relatively low invasion potential and anchorage-independent growth ability, however, higher expression of ITGB4 and ITGA6 in CMT-U229 may also contribute to its high growth rate by promoting cell survival. CMT-U229, with high expression of many genes involved in cell cycle, immune related (cytokine, complement cascade) and cytoskeletal/Rho-GTPase signalling could serve as an interesting model to further investigate the role these signalling pathways in canine mammary carcinogenesis.

Cell line P114

Cell line P114 was isolated from a highly malignant canine primary anaplastic carcinoma and has been reported to carry wild type p53 [13]. The proliferation assay revealed a relatively fast doubling time (22 hours) for this cell line (Fig. 1). In addition, the *in vitro* invasion and soft agar colony formation assays indicated P114 as the most tumourigenic of the three cell lines. The histogenesis of the anaplastic carcinomas is not very clear, but they are believed to have an epithelial origin [36] and indeed high expression of CK18 and CK8 transcripts (epithelial markers) in P114 compared to non epithelial CMT-U335 was evident from our microarray data. This suggests that the cell line P114 may have retained key characteristics of its tumour of origin.

Additionally, P114 has also showed differentiation ability when cultured in matrigel matrix by demonstrating branching morphogenesis (Fig. 2).

Gene expression profiles revealed a clear set of genes that are differentially expressed in P114 compared to the other two cell lines. Many genes involved in the Wnt-signalling pathway were identified when these differentially expressed genes were subjected to the pathway analysis. Wnt-signalling is driven by secreted Wnt ligands and their frizzled receptors and is very important in early embryonic development and adult tissue maintenance [37]. In many tissues aberrant activation of Wnt-signalling is associated with malignancy [38]. Mutations in key regulators of Wnt-signalling, namely APC, beta-catenin and TCF are the main causes of active Wnt-signalling in colorectal cancer (reviewed in [39]). Similar mutations are uncommon or not yet reported in the tumourigenesis of breast and other tissues. Among the breast cancer studies, there are many reports of high nuclear beta-catenin immunoreactivity, which is a hallmark of active Wnt-signalling [38, 40]. In this study, P114 seems to have a highly active Wnt pathway supported by the overexpression of many activators or positive regulators of Wnt-signalling (CTNNB1, CSNK1A1, DDR1, CHD1L, OPN and CCND1) and down regulation of many Wnt-signalling repressors (DKK3, SFRP1). An activated canonical Wnt pathway mediated by beta-catenin/TCF activity in P114 was further confirmed using a TCF-Luciferase reporter assay (manuscript under preparation). Active beta-catenin has been reported to regulate cadherin mediated cell-cell adhesion as well as cell proliferation [41]. Loss of expression of Wnt antagonists like SFRP1 and DKK3 has been identified in many types of cancers and is known to be associated with aggressiveness [42-44]. P114 indeed showed potent anchorage-independent growth ability and was the most invasive cell line in our study based on transwell invasion assay. Constitutively active Wnt-signalling could downregulate E-cadherin and make cells more invasive, although this may not be the cause for high tumourigenicity of P114 because E-cadherin expression was not significantly lower in P114. Another possible explanation could be through the Wnt-signalling target gene osteopontin (OPN) [45] which is also overexpressed in P114. OPN can make tumours highly tumourigenic by regulating the expression of urokinase plasminogen activator (uPA) through phosphatidylinositol 3-kinase/akt signalling [46]. OPN is also

overexpressed in P114, and there is also a higher expression of uPA mRNA in P114 compared to other two cell lines (unpublished data).

Wnt-signalling has a major role in breast cancer, as evidenced by observed high nuclear beta-catenin immunoreactivity in 60% of analyzed human breast tumours [40] and loss or reduced expression of Wnt antagonist SFRP1 80% of human breast carcinomas [47]. Therefore, the highly tumourigenic P114 cell line with its active Wnt pathway could be a valuable model to study the role of the Wnt pathway in mammary tumourigenesis and progression.

Cell line CMT-U335

Cell line CMT-U335 was isolated from a rarely occurring (< 1%) primary canine mammary osteosarcoma [36], an aggressive tumour, expressing bone components, extra cellular matrix (ECM) proteins including vimentin and display weak or no staining for epithelial cytokeratins [48]. Gene expression profile of CMT-U335, the slowest growing cell line of the three cell lines revealed a large number of differentially expressed genes compared to P114 and CMT-U229 (Supplementary Table 1), indicating that it is more distinct from the other two cell lines. Enrichment of the gene expression data by pathway analysis identified overexpression of many genes involved in integrin signalling. Genes responsible for active Wnt-signalling and cell cycle were expressed at lower levels in CMT-U335, which may explain its slower growth rate.

ECM, through its cell surface integrin receptors, exerts enormous control over the behaviour of normal as well as cancerous cells. Specific combinations ECM and integrin interactions control cell motility, shape, growth and survival [49]. The highly expressed Integrin-β-5 (ITGB5) in CMT-U335 may have stimulated the autocrine TGF-β-signalling leading to the deposition of ECM proteins [50]. This hypothesis is further supported by the high expression of collagens (COL1A2, COL4A1, and COL12A1) in this cell line. COL1A2 is normally expressed in supportive or connective tissue and plays a key role in osteogenesis [51]. Overexpression of COL1A2 or other ECM proteins involved in actin cytoskeleton assembly and cell-cell or cell-matrix interaction (VCL, ARPC5) indicates possible aberrant integrin signalling in this cell line. Additionally, epithelial cytokeratin (CK8/CK18) transcripts were expressed at a lower level and osteogenic (COL1A2, OGN) was expressed at a higher level indicating the preserved mesenchymal characteristics of its primary tumour. CMT-U335 formed fewer colonies when

grown in soft agar and invaded at a slower rate through matrigel coated transwells. In addition, CMT-U335, representative of a rarely occurring primary tumour which overexpresses many integrin signalling genes, would be an interesting mammary osteosarcoma model for in vitro studies.

Cross species comparison of pathway profiles

Cross species comparison can be a powerful biological sieve to select important oncogenic pathways which may have evolutionary significance. Comparing the pathway profiles of canine mammary cell line gene set, the human breast cancer intrinsic gene set and human breast cancer cell line gene set identified four of the five major signalling pathways as common across the datasets. These results stress the importance of Wnt, integrin, angiogenesis and cytokine/ chemokine signalling in mammary cancer of dogs and humans. Wnt-signalling is already known for its role in human mammary development and carcinogenesis (reviewed in [38]). Angiogenesis plays a central role in both local tumour growth and distant metastasis and its importance in breast cancer development and progression has been documented by many authors [52]. Cytokine/chemokine signalling have been implicated in breast cancer, but the signalling network as a whole is not well understood in relation to the etiology of breast cancer. The importance of integrin signalling in breast cancer has been established by many authors [50, 53]. Parkinson's disease in canine gene set and Alzheimer's disease signalling in human breast cancer gene set were identified among the top five pathways. Since these pathways are less likely to be associated with breast cancer and were not common across the three gene sets, they can be considered as of trivial significance for mammary carcinogenesis.

Conclusion

This study identified Wnt-signalling, integrin signalling, cell cycle, alternative complement cascade and cytokine/Rho-GTPase signalling as main pathways regulated differentially in CMTs. Cross species comparison of pathway profiles identified four of the five major deregulated signalling pathways are common in human and canine data sets indicating molecular similarity of the disease across species. Further studies correlating an individual cell line phenotype with its gene expression signature may facilitate in annotating large groups of differentially expressed

unannotated genes. These characterized CMTs with their unique gene expression and pathway profiles are valuable tools to prioritize biological pathways for a detailed study. In the light of the high incidence of mammary cancer in dogs these cell lines are a valuable tool to develop and to test new pathway specific caner therapeutics. These results are also the basis for further characterization of canine mammary carcinomas.

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Chapter 4

CDC2 mediated overexpression of RGC32 and BF stimulates canine mammary tumor cell proliferation

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(Submitted)

Abstract

Tumors often exhibit amplified cyclin dependent kinase activity and associated deregulation of the cell cycle. Response gene to complement 32 (RGC32) was initially identified as a substrate and regulator of cell division cycle 2 (CDC2) and its overexpression in many human cancers has been reported to be involved in cell cycle deregulation. Contradicting reports on the function of RGC32 have been suggested to be related to a tissue specific variation in RGC32 function. Factor B (BF) is a key regulator of the alternative complement pathway and its overexpression in tumors is largely associated with inflammatory response. We identified overexpression of RGC32 and BF in a canine mammary cell line (CMT) with rapid growth rate and amplified CDC2 kinase activity when compared to slow growing CMTs with lower CDC2 activity. In this study, we used a combination of chemical inhibition of CDC2 and RNA interference to gain further insight into the cause of overexpression of RGC32 and BF in CMT. Chemical and siRNA mediated inhibition of CDC2 activity resulted in some 80% inhibition of cell proliferation and a significant transcriptional repression of RGC32 and BF. Individual depletion of RGC32 or BF mRNA resulted in significant cell growth arrest. Expression of RGC32 and BF showed a high correlation when analyzed in a panel of mammary tumors, cell lines and normal tissues. These data indicate that overexpression of RGC32 and BF in mammary tumors is co-regulated by CDC2 and they may serve as markers for increased CDC2 activity or associated high proliferative index.

Introduction

Breast cancer is the most common cancer of women, comprising 23% of all female cancers [1] and the incidence of mammary tumors in dogs is even more frequent than in women [2]. Mammary cancers in both species are ovarian hormone dependent and are similar in many aspects [2-8]. To study the tumor biology and translational cancer therapeutics, especially gene targeting methods, homologous canine mammary tumor cell lines (CMTs) are useful models. Recently, we characterized CMTs, which are distinct with respect to their phenotype and primary tumors of origin, using cDNA microarray [9]. Among the identified deregulated genes pertaining to the phenotypes of cell lines, namely growth rate and *in vitro* invasiveness, BF and RGC32 were highly overexpressed in the cell line (CMT-U229) with highest growth rate.

BF, synthesized in liver and at extrahepatic sites such as endothelial, epithelial and mesenchymal cells, is a 93-kDa single-chain glycoprotein required for the initiation and propagation of an alternative complement pathway [10]. Activation of this pathway leads to the assembly of membrane attack complex (C5b-9) which primarily induces cell lysis, and at sublytic concentrations is reported to activate cell cycle through RGC32 in various cell types [11, 12]. BF is also known to induce proliferation of B-lymphocytes through a mechanism similar to the B-cell growth factor pathway [13]. Expression of BF and other complement genes in human mammary gland have been reported by many authors [14, 15]. Their role in cell cycle or other pathways in the mammary gland, excluding inflammatory processes is unknown.

RGC32 was initially identified in primary rat oligodendrocytes (OLG) as a gene induced by sublytic concentrations of C5b-9 [12]. Overexpression of RGC32 in rat OLG and OLGxC6 glioma cell hybrids was associated with increased DNA synthesis in response to serum growth factors or C5b-9 [12]. Later it was found that RGC32 forms a complex with CDC2 and increases its kinase activity [11]. Overexpression of RGC32 protein was observed in colon, prostate, bladder, breast, lung and other digestive tract tumors and it co-localized with proliferation marker Ki-67 in malignant epithelial cells [16]. From these studies it is evident that RGC32 is a cell cycle stimulator and a putative oncogene. A recent study identified RGC32 as a direct target of p53 and

reported that it functions as a tumor suppressor in glioma cell lines [17], a discrepancy its author has explained by speculating that RGC32 may function differently among different cell types.

Each stage of the cell cycle is tightly controlled by cyclins, cyclin dependent kinases (CDKs) and their inhibitors [18]. CDK activity is regulated by phosphorylation and dephosphorylation by classical association /dissociation of its regulatory cyclin subunits or by specific inhibitor molecules such as $p21^{Waf}$ [18, 19]. Among different CDKs, CDC2 and its regulatory subunit cyclin B1 (*CCNB1*) are believed to be indispensable for G2/M transition of the cell cycle. Recent reports suggest that CDC2 is also able to compensate for CDK2 activity during G1/S transition of cell cycle emphasizing its predominant role in cell cycle regulation [20]. Deregulation of the cell cycle is a common and well documented phenomenon in cancer [21]. Currently, CDKs are also being considered as novel targets for development of anticancer drugs (reviewed [22]). Additionally, an increasing number of genes have been found to encode for substrates or activators or effectors of CDKs [11, 23]. Although the exact function of RGC32 is controversial, it is clearly a candidate gene associated with CDC2 activity and tumorigenesis.

With an exception of one study reporting immunoreactive RGC32 in human breast tumors, there are no reports of investigations of the role of RGC32 and/or BF in mammary tumorigenesis [16]. Yet the overexpression of BF and RGC32 in a fast growing CMT-U229, suggest that they may have a role in cell cycle progression and mammary tumorigenesis. After comparing the CDC2 kinase activity in mitotic cell extracts of three CMTs in order to investigate its relation with RGC32 gene expression, we analyzed the expression of RGC32 and BF in normal and tumorous canine mammary tissue. A specific inhibitor of CDC2 activity and siRNA technology was used to investigate the presumed crosstalk between CDC2, RGC32 and BF and their role in proliferation of CMTs.

Methods

Cell Culture

The cell line CMT-U335 was isolated from a canine mammary osteosarcoma, CMT-U229 from a canine mammary atypical benign mixed tumor and P114 from a highly malignant canine

anaplastic carcinoma. Isolation and characterization of these three cell lines were reported earlier [24, 25]. Cell lines were grown in culture medium (DMEM-F12 (Invitrogen, The Netherlands) supplemented with 10% fetal calf serum (PAA Laboratories, Austria)) at 37^0C in a humidified atmosphere of 5% CO_2. All experiments were performed using cell lines at early passages (3-22 passages). For CDC2 inhibition studies, media containing different concentrations (10-2000 nM) of CDC2 specific inhibitor [26] CGP-74514A-HCl (CGP) (Sigma, The Netherlands) was prepared in culture medium and cells were treated 24 hours after plating.

RNA interference

Double stranded RNAs of 21 nucleotides (Stealth^{TM}) were obtained from Invitrogen. The sequences of siRNAs used in this study are listed in Table 1. Transfection was performed using the Magnet Assisted Transfection (MATra) technique (IBA BioTAGnology, The Netherlands) in combination with lipofectamine2000™ (Invitrogen, The Netherlands) as described earlier [27]. Medium-GC RNAi negative control (Invitrogen, The Netherlands) was used as a control for sequence independent effects following siRNA transfection. Cells were plated in 6-well plates (BD Biosciences, The Netherlands) at a density of 10^4 cells / well. After 24 hours of incubation, cells were transfected with gene specific siRNA duplexes or control siRNA duplexes. After transfection, the transfection medium was replaced with culture medium. Experiments were performed in triplicates and repeated 4-5 times.

Cell proliferation assay

Cells were seeded in 6-well plates at a density of 10^4 cells per well in culture medium. After 24 hours, cells were washed with Hank's Balance Salt Solution (Invitrogen, The Netherlands) and subjected to treatments (siRNA transfection or CDK inhibitor). Cell proliferation rate was estimated 48-72 hours after treatments using [^{3}H]-Thymidine incorporation as described earlier with minor modifications to the original protocol [28]. To each well, [^{3}H]-Thymidine (25.0 Ci/mmol) (GE Healthcare, UK) was added to a final concentration of 0.1 µCi/ml. After incubation for 4.5 hours (37^0C; humidified atmosphere ; 5% CO_2), cells were washed and lysed in 0.1 ml of 0.2 % SDS after 15 min followed by addition of 400 µl water. Two milliliters scintillation fluid

was added to 400 µl of cell lysate and incorporation of [^{3}H]-Thymidine was measured by a liquid scintillation analyzer (TRI-CARB 2900TR, Packard).

CDC2 kinase assay

Cells (2x10^6 cells / flask) were plated in 75 cm^2 CELLSTAR® tissue culture flasks (Greiner Bio-One, The Netherlands) in 10 ml culture medium. After 24 hours, cells were mitotically synchronized by adding nacodazole to a final concentration of 1 µg/ml. Only loosely adherent mitotic cells were harvested after 12-20 hours by carefully removing medium and cell debris. Mitotic cells were pelleted by centrifuging at 200 x g for 10 minutes at 4^{0}C followed by rinsing in cold PBS. Cell pellets were re-suspended in 3-5 pellet volumes of cold extraction buffer (50mM Tris-HCl pH 7.4; 250mM NaCl; 1mM EDTA; 50mM NaF; 1mM DTT; 0.1% Triton® x-100; 10µM leupeptin; 100µg/ml aprotinin; 0.5 mM PMSF) and lysed by brief sonication. Clear protein solution was obtained by centrifuging at 100,000 x g for 1 hour at 4^{0}C. Total protein concentration was determined by using a Lowry-based, DC-Protein assay system (Bio-Rad, The Netherlands). CDC2 kinase activity in the mitotic cell lysates was analyzed using SignaTECT® CDC2 kinase assay system (Promega, The Netherlands) according to the manufacturer's protocol. This assay system uses biotinylated peptide substrate derived from histone H1 which is highly specific for CDC2. The radiolabelled, phosphorylated substrate recovered from the reaction mix was placed in scintillation vials. The activity of the substrate was measured using liquid scintillation counter (PerkinElmer, The Netherlands) and the kinase activity is expressed as pmol of ATP/min/µg protein.

Western blot analysis

Cells were homogenized in 250 µl (for a 6-well plate well) RIPA buffer containing 1% Igepal, 0.6mM PMSF, 17µg/ml aprotinin and 1mM sodium orthovanadate (Sigma, The Netherlands) for 30 minutes on ice. After centrifugation at 10000g for 10 min, clear supernatants were collected. Protein concentrations were determined as explained earlier and western blotting was performed according to the previously published protocol [29]. Nitrocellulose membranes containing proteins were probed using primary antibodies by incubating overnight at 4^{0}C. Mouse anti-human CDK1 monoclonal antibody (StressGen Biotechnologies Corp, CA) was used at a dilution of

1:2000 in TBST (0.1% tween) with 1 % BSA (Sigma, The Netherlands). Rabbit anti-human (Phospho-Thr161) CDK1 (Cell Signaling Technology, MA) was used at a dilution of 1:1000 in TBST with 1%BSA. Mouse anti-human β-actin (NeoMarkers, CA) was used as the loading control. After washing, the membranes were probed with anti-mouse or anti-rabbit HRP secondary antibodies (R&D systems/Westberg b.v., The Netherlands) in TBST with 1% BSA for 1 hour at room temperature. Membranes were exposed to Kodak BioMax light-1 films (Sigma, The Netherlands).

Table 1. *Primer pairs used in this study for qRT-PCR and siRNA duplexes used for gene knockdown: Tm = optimum annealing temperature of qRT-PCR primer pairs.*

Gene	Accession Number	Sequence (5′-3′)	Primer / siRNA	Tm (^{0}C)	Product size (bp)
CDC2	XM_546115	TCAGTCTTCAGGATGTGCTTATG	Forward	61	102
		GACCAGGAGGGATGGAATCT	Reverse		
CCNB1	XM_846071	GTGAATGGACACCAACTCTACA	Forward	58	144
		AGGCGGCATACTTGTTCTTG	Reverse		
RGC32	XM_846804	CATCTCTGTCGGCTCTGGTAG	Forward	58	99
		AAGTTCTGGGTCCTTTCATCAT	Reverse		
BF	XM_844892	AGGGACACGAAACCTGTATG	Forward	62	106
		ACGCTGACCTTGATTGAGTG	Reverse		
*CDC2	XM_546115	GGGAUUCAGAAAUCGAUCAACUCUU		NA	NA
		AAGAGUUGAUCGAUUUCUGAAUCCC			
*RGC32	XM_846804	GAAAGCCAAACUAGGAGACACGAAA		NA	NA
		UUUCGUGUCUCCUAGUUUGGCUUUC			
*BF	XM_844892	CACACUGUUUCACAGAGAAUGAUCA		NA	NA
		UGAUCAUUCUCUGUGAAACAGUGUG			

*Note: * siRNA duplexes used in this study. NA= not applicable.*

RNA isolation and Real time quantitative-PCR (qRT-PCR)

Total RNA from canine mammary carcinomas and cell lines was isolated using RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Synthesis of cDNA was carried out from 1.5 µg total RNA in 60 µl reaction volumes using the iScript^{TM} cDNA synthesis

kit according to the manufacturer's protocol (Bio-Rad, The Netherlands). Primers (Table 1) were designed using primer select software of DNA star (Madison, WI) according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product. HPRT, GAPDH and RPS19 genes were used as the non-regulated reference genes for normalization of target gene expression [27]. qRT-PCR was performed as described previously using Bio-Rad MyIQ detection system (Bio-Rad, The Netherlands) with SYBR green fluorophore. Data analysis was carried out using the pairwise fixed reallocation and randomization test incorporated in the software program REST-XL [30] at 5% level of significance. Every experiment was performed in triplicate and the average values from 4-5 independent experiments were used for data analysis.

Statistical analysis

Mean values from 4-6 independent experiments of treatment and control were compared for significant difference using student's t-test and expressed as percentage of control values. Basal gene expression in each cell line was quantified using cDNA derived from 6 independent biological replicates. Statistical significance of mean difference in gene expression (for each gene) between CMTs was carried out using the pairwise fixed reallocation and randomization test incorporated in the software program REST-XL [30] at 5% level of significance. Relative expression of every gene in each cell line, normalized to that of cell line CMT-U335 is presented.

Results

RGC32 and BF are differentially expressed in CMTs

Using qRT-PCR, the previously identified overexpression of RGC32 and BF transcripts in the rapidly growing cell line CMT-U229 using canine cDNA microarray [9] were confirmed and depicted relative to that of the slowest growing cell line CMT-U335 (Fig.1A). Cell line P114 showed an intermediate expression of RGC32. CDC2 expression was not different between cell lines, but the associated CCNB1 was significantly overexpressed in cell line P114 and CMT-U229 (Fig. 1A). BF was significantly upregulated only in the CMT-U229 cell line.

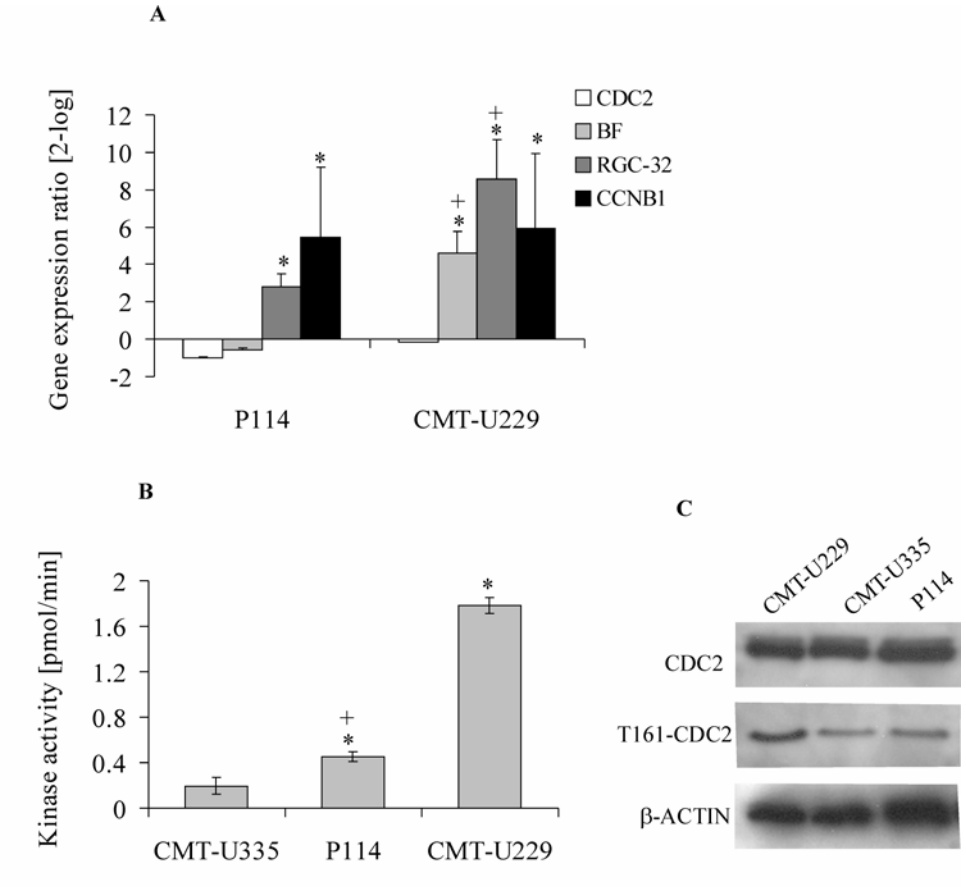

Figure 1. A: *Basal gene expression in canine mammary cell lines depicted as the ratio to that of slow growing cell line CMT-U335. * $p < 0.001$ versus corresponding gene expression in CMT-U335. + $p < 0.001$ versus corresponding gene expression in P114. RPS-19 and HPRT were used as reference genes for normalization of target gene expression.* ***B****: Basal CDC2 kinase activity in mitotic cell lysates of CMTs estimated using CDC2 kinase assay kit. 5µg of mitotic cell lysate from each cell line was assayed for 1 minute using 50µM ATP and 1µCi of [γ-32 P] ATP/reaction.* ***C:*** *Mitotic CDC2 kinase (CDC2 and Phospho (T161)-CDC2) levels in nacodazole synchronized cell lysates of canine mammary cell lines were determined by western blotting. β-actin is used as the loading control.*

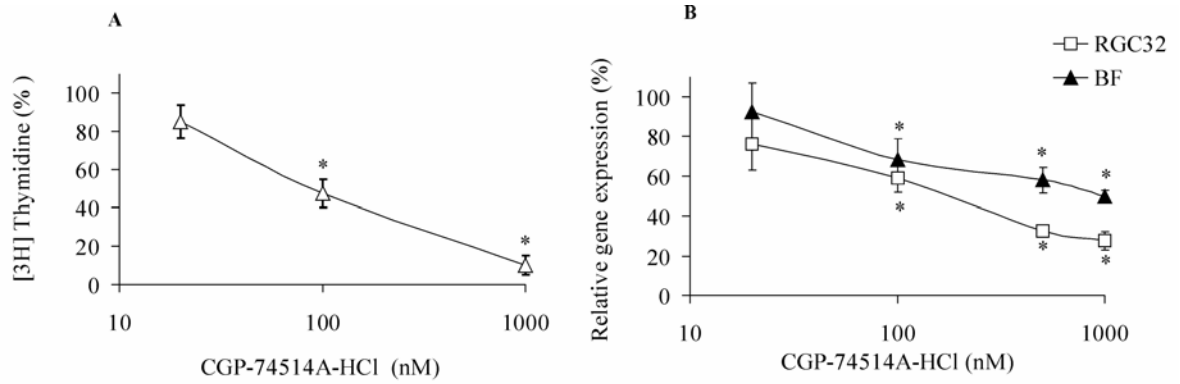

Figure 2. *CDC2 inhibition:* ***A:*** *Cell line CMT-U229 was treated with different concentrations of CGP-74514A-HCl for 48 hours and [^{3}H]-Thymidine incorporation was used as a measure of cell proliferation rate. Results are presented as the ratio (%) of non-treated control cells.* ***B:*** *CMT-U229 was treated with different concentrations of CGP-74514A-HCl for 24 hours and gene expression of RGC32 and BF were estimated. Quantified gene expression is presented as that ratio to that of non-treated cells. * $p < 0.05$ versus non-treated cells.*

CMTs show a difference in mitotic CDC2 kinase activity in vitro

RGC32 is reported to bind to CDC2 and increase its kinase activity during G2-M transition of cell cycle. CCNB1, the regulatory subunit of CDC2 is essential for CDC2 kinase activity. Changes in expression of CCNB1 and RGC32 may influence the activity of CDC2 in CMTs. Therefore, the CDC2 activity was estimated in cell lysates of G2/M synchronized CMTs using an *in vitro* assay. Cell lysates were also subjected to western blot analysis for active phospho-CDC2 (T161) and CDC2 proteins. Interestingly, cell lysates of CMT-U229 displayed a significantly higher CDC2 kinase activity compared to other cell lines. Cell line P114 also showed significantly higher CDC2 kinase activity when compared to that of CMT-U335 (Fig. 1B). Even though there was no difference in CDC2 protein level between cell lines, CMT-U229 displayed relatively higher levels of phospo-CDC2 (T161) (Fig. 1C).

CDC2 inhibitor dose dependently inhibits the growth rate of CMT-U229

To confirm that the increased CDC2 activity was responsible for the high growth rate of CMT-U229, the kinase activity was inhibited using the selective CDC2 inhibitor CGP. In cell lysates of

CMT-U229, CGP inhibited CDC2 kinase activity dose dependently (Suppl Fig. 1). In addition CGP treatment resulted in accumulation of cells at G2/M phase (not shown) similar to earlier studies (31). Cell proliferation rate as measured by [^{3}H]-Thymidine incorporation decreased dose dependently upon CDC2 specific inhibition (Fig. 2A). The inhibition of CDC2 kinase activity also caused a dose-dependent inhibition of RGC32 and BF mRNA expression (Fig. 2B).

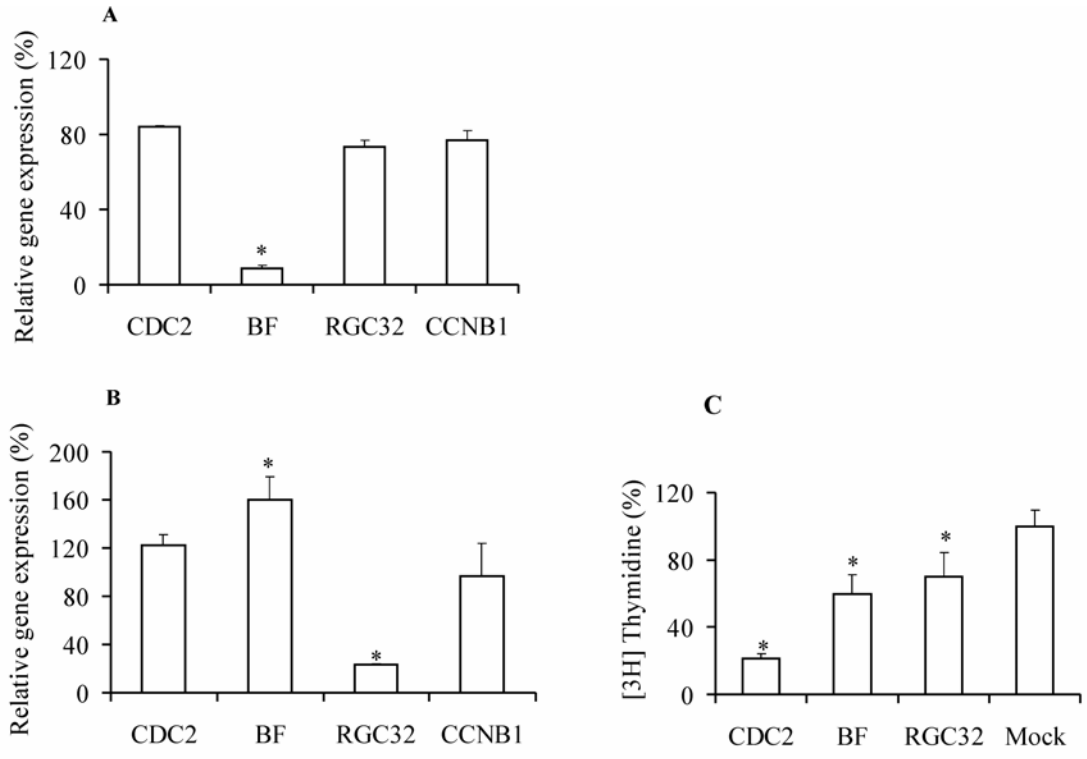

Figure 3. *Specific depletion of A: BF and B: RGC32 and their effect on gene expression after 72 hours of siRNA transfection. Quantified gene mRNA levels are presented as the ratio to that of control siRNA treated cells. C: Effect of specific depletion of CDC2, BF and RGC32 genes for 72 hours on cell proliferation rate of CMT-U229. Proliferation rate is expressed as percentage [^{3}H]-Thymidine incorporation to the control siRNA treated cells.* * $p < 0.05$.

siRNA depletion of RGC32, BF and CDC2 inhibit cell proliferation

Next, the effect of selective depletion of these genes on proliferation of the cell line CMT-U229 was investigated. siRNAs targeting RGC32 and BF were effective in significantly depleting their

expression (Fig 3A and 3B). RGC32 depletion did not alter the expression of CDC2 and CCNBI significantly but slightly stimulated the expression of BF 72 hours after transfection. BF depletion did not influence the expression of CDC2, RGC32 or CCNB1 but showed approximately 40% growth inhibition compared to mock treated cells (Fig. 3C). RGC32 depleted cells showed more than 25% lower [^{3}H]-Thymidine incorporation compared to mock transfected cells. The highest growth inhibition (~80%) was noticed after CDC2 depletion (Fig. 3C) which is in agreement with chemical (CGP) inhibition of CDC2. Similar to CGP treatment, CDC2 knockdown resulted in accumulation of cells at G2 phase (not shown).

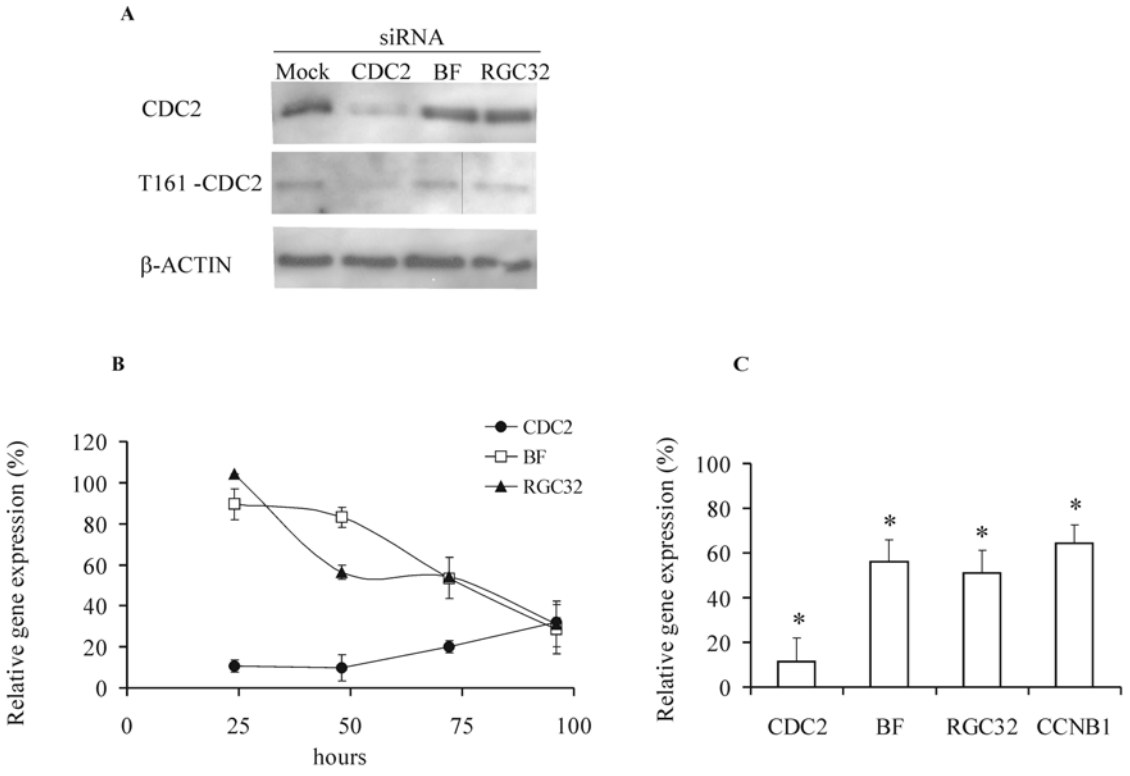

Figure 4. *siRNA mediated depletion of CDC2.* ***A:*** *Western blot analysis of siRNA transfected CMT-U229 cell line lysates 72 hours after transfection. β-actin was used as the loading control;* ***B:*** *Downregulation of BF and RGC32 follow CDC2 depletion time dependently.* ***C:*** *CDC2 specific siRNA down regulates BF, RGC32 and CCNB1 (72 hours after siRNA transfection); * $p < 0.05$ versus expression in control siRNA transfected cells.*

siRNA mediated depletion of CDC2 inhibits the expression of RGC32 and BF

CDC2 siRNA was very effective in depleting its protein and mRNA levels for up to 96 hours (Fig. 4A and 4B). The mRNA levels of BF and RGC32 decreased significantly and followed the CDC2 mRNA expression in a time dependent manner (Fig. 4B). At 72 hours, when we observed a significant depletion of CDC2 protein, RGC32 and BF transcripts were depleted up to 50% when compared to mock transfected cells. Additionally, CCNB1 expression was also suppressed at 72 hours of CDC2 depletion (Fig. 4C).

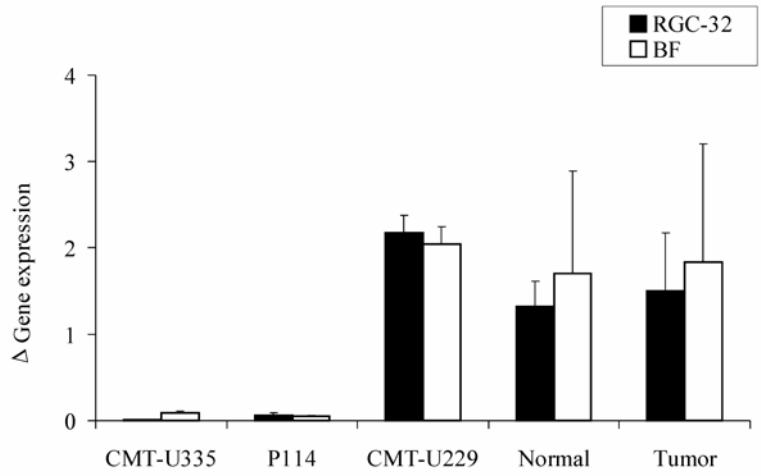

Figure 5. *RGC32 and BF expression pattern in canine mammary tissue and cell lines. Values on Y axis are the reference gene-normalized starting quantities (2-log) which are divided by a common reference value.*

Expression of RGC32 and BF in canine mammary tumors and cell lines

There are no studies addressing the significance of the expression of RGC32 and BF transcripts in normal mammary tissue and tumors. The expression of these targets in a panel of 8 normal canine mammary tissues and 10 tumors were investigated. A wide variation in expression of RGC32 and BF was observed in both normal mammary and tumor tissues. There was no statistically significant difference between expressions of these genes between normal tissue and tumors (Fig. 5). However, we observed a significant correlation ($R^2 = 0.70$; $p<0.01$) between the expression of RGC32 and BF in canine mammary tumors, normal tissue and cell lines.

Discussion

Canine mammary tumor cell lines show a clear positive correlation between their growth rate and expression of CCNB1, RGC32 and BF genes. The cell line CMT-U229 with shortest doubling time expresses CCNB1, RGC32 and BF transcripts at a very high levels (>4-fold) compared to the cell lines with slower growth rate. This led us to suspect CCNB1, RGC32 and BF as candidate genes responsible for high growth rate of CMT-U229. CCNB1 is the regulatory subunit of CDC2 which is a key cyclin dependent kinase involved in the G2-M transition of the cell cycle [31]. Overexpression of CCNB1 is frequently observed in many human cancers and is associated with higher proliferative index [32]. Similarly, RGC32, a reported activator of CDC2 is also found to be overexpressed in a variety of human malignancies [11, 16]. However, the mechanism of overexpression is not clearly understood. A recent study identified RGC32 as a direct transcriptional target of tumor suppressor p53 and the level of RGC32 mRNA tended to decrease in tumors with p53 mutations [17]. The cell lines CMT-U229 and CMT-U335 with the highest and lowest expression of RGC32, respectively, both carry mutations in the DNA binding domain of p53 whereas, P114 with intermediate RGC32 expression carries wild type p53 [25]. Therefore it is highly unlikely that p53 mediates transcriptional regulation of RGC32 in CMTs.

The role of BF in cell cycle progression or tumorigenesis is less known. BF is a key activator of an alternative complement pathway which leads to assembly C5b-9 [33]. C5b-9 is reported to induce RGC32 expression through the ERK pathway leading to an increased CDC2 activity [34]. Therefore, we hypothesized that high expression of BF would be the cause of increased RGC32 expression through the C5b-9 complex mediated pathway. In addition to overexpression of CCNB1, the high RGC32 expression would then result in an amplified CDC2 activity explaining the high growth rate of CMT-U229. From our experiments it can be concluded that this is clearly not the case (see below).

The CDC2 activity is maximal during mitosis and appeared also to be maximal in CMT-U229 cells after mitotic synchronization in comparison to the other cell lines. During the cell cycle the kinase activity of CDC2 is dependent on its stimulatory (T161) and inhibitory (T14/Y15) phosphorylation status [35]. Higher level of phospho-(T161)-CDC2 in mitotic cell

lysates of CMT-U229 may partially explain its high kinase activity, although lack of inhibitory phosphorylation could as well lead to higher CDC2 kinase activity (not tested).

RGC32 overexpression mediated smooth muscle cell proliferation was abolished by butyrolactone, a known CDC2 inhibitor [11]. In a similar approach, inhibition of CDC2 activity by CGP inhibited the cell proliferation of CMT-U229 significantly. Interestingly, expression of both RGC32 and BF were inhibited dose dependently with the maximum inhibition up to 50%. These results indicate an existence of a CDC2 activity dependent mechanism of RGC32 and BF transcription in CMTs.

In order to get further insight in the crosstalk between CDC2, RGC32 and BF, their mRNAs were depleted using specific siRNAs. Similar to CGP mediated effect, CDC2 knockdown resulted in significant growth arrest and transcriptional repression of RGC32 and BF in CMT-U229. Inhibition of the expression of RGC32 and BF resulted in partial growth inhibition that however, is much less than the effect seen after CDC2 inhibition. Our data clearly suggest a CDC2 activity dependent transcription of RGC32 and BF. As mentioned earlier, if RGC32 is regulating the kinase activity of CDC2, then its depletion (siRNA) would inhibit expression of BF as observed in CDC2 inhibition using siRNA or CGP. Instead, RGC32 depletion had a slight stimulatory effect on BF expression. These data suggest that RGC32 may not regulate the kinase activity of CDC2 in CMTs in contrast to what has been reported in others cell types [11]. Similarly, BF depletion did not influence RGC32 transcription. However, individual depletion of these targets clearly inhibited cell proliferation. There is no evidence to support our initial hypothesis that BF and RGC32 are upstream of CDC2. Instead, CDC2 kinase activity directly or indirectly drives the expression of BF and RGC32 and thus mediates cell proliferation of CMTs. Additionally, expression levels of CDC2 and CCNB1 were monitored after individual knockdown of RGC32 and BF transcripts. There was no significant change in the expression of these genes, which excludes the possibility that RGC32 and BF regulate the expression of CCNB1 and CDC2 and thus stimulate cell proliferation. BF knockdown resulted in a significant inhibition of cell proliferation (~40%). However, the exact mechanism of RGC32 and BF mediated cell proliferation is not clear. Cleaved active Bb fragment of BF is known to bind receptor of B-cell

growth factor and stimulate B-cell proliferation through an unknown mechanism [36]. In a similar way, BF may act as endogenous growth factor in CMTs leading to their proliferation.

The family of cyclin dependent kinases comprises two groups based on their role in cell cycle progression and transcriptional regulation. Members of cell cycle dependent kinases are known to regulate core cell cycle events and the transcriptional CDKs regulate target gene transcription by phosphorylation of the COOH-terminal domain (CTD) of RNA polymerase-II [22, 37]. Even though CDC2 is generally considered as a cell cycle CDK, recent evidences indicate its ability to phosphorylate CTD element of RNA polymerase-II *in vitro* [38]. Therefore, through a similar mechanism CDC2 is likely to regulate the expression of RGC32 and BF. In CMTs, the expression pattern of RGC32 and BF correlate very well with the kinase activity and specific inhibition of CDC2 kinase activity inhibits the expression of BF and RGC32 consistently in addition to cell growth inhibition. CDC2 inhibition had no effect on expression of other unrelated genes (MMP2 and RAC1/ RAC1B) which rules out the possibility of general transcriptional repression (not shown). From these results, it is clear that RGC32 and BF genes are downstream targets of CDC2. The increased plasma BF concentrations of BF as seen in breast cancer patients [15] might also be the result of enhanced CDC2 activity rather than complement activation.

In order to establish the biological significance of the expression of RGC32 and BF in the mammary gland, their expression in a panel of normal tissues and tumors was analyzed. The expression of these genes in normal tissues and mammary tumors were not statistically different. From the cell line expression data it is clear that there is a large variation in expression of RGC32 and BF in cell lines depending on their CDC2 kinase activity. Tissues analyzed in this study were not selected based on their CDC2 kinase activity or proliferation characteristics, which may explain the large variation in RGC32 and BF expression. In other words, the expression pattern of RGC32 and BF may only indicate the activity of CDC2. A regression line fitted between the expression values of RGC32 and BF in the canine mammary tumors and cell lines showed a very strong correlation between their expressions, indicating co-regulation of RGC32 and BF in canine mammary tumors. CDC2 overexpression is often observed in tumors. However, kinase activity of CDC2 depends on its (inhibitory or stimulatory) phosphorylation status rather than its expression

level. Therefore, overexpression of CDC2 as observed in tumors may not always indicate its increased activity or high proliferative index. In this context, overexpression of RGC32 and BF in mammary tumors may serve as valuable markers for increased CDC2 activity and associated proliferative index.

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Chapter 5

Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch

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Abstract

Endogenous progesterone and synthetic progestins may induce hypersecretion of growth hormone (GH) of mammary origin, hyperplastic ductular changes in the mammary gland, and the development of cystic endometrial hyperplasia (CEH) in dogs. It was investigated whether progestin-induced mammary GH plays a role in the pathogenesis of CEH in the bitch. During 1 year, bitches with surgically excised mammary glands and healthy control bitches received medroxyprogesterone acetate (MPA). Before and afterMPAtreatment, uterine and mammary tissues were collected for histological, immunohistochemical, and RT-PCR examination.

After MPA administration, the mammary tissue in the control dogs had differentiated into lobuloalveolar structures andCEHwas present in all uteri of both dog groups. In theMPA-exposed mammary tissue of the control dogs, GH could only be demonstrated immunohistochemically in proliferating epithelium. After treatment with MPA the dogs of both groups had immunohistochemically demonstrable GH in the cytoplasm of hyperplastic glandular uterine epithelial cells. RT-PCR analysis of the mammary gland tissue after MPA administration demonstrated a significant higher GH gene, and lower GHR gene expression than before treatment. In the uterus, the expression of the gene encoding for GH was significantly increased in the mastectomized dogs, whereas in the control dogs the expression of the gene encoding for insulin-like growth factor-I had significantly increased with MPA administration. MPA treatment significantly downregulated PR gene expression in the uterus in both dog groups. These results indicate that progestin-induced GH of mammary origin is not an essential component in the development of CEH in the bitch.

Introduction

Cystic endometrial hyperplasia (CEH) is a common disorder of the canine uterus and may result in infertility [1–3]. The condition begins with endometrial glandular hyperplasia and progresses to cystic transformation of the glands. When CEH is accompanied by a bacterial infection, the disorder is called CEH-endometritis or, if the cervix is closed, pyometra [4–8]. This systemic disease may result in death due to toxaemia, glomerulonephritis, and peritonitis [9]. The pathogenesis of CEH remains incompletely understood, but progestins play an important role in the development of CEH in bitches [10]. Consequently, CEH is frequently seen in bitches treated repeatedly with progestins for oestrus prevention [11–13]. Cystic endometrial hyperplasia may also develop spontaneously during the luteal phase of the oestrous cycle of middle-aged and elderly bitches, i.e. bitches that repeatedly have been under the influence of high concentrations of endogenous progesterone [4].

In dogs, endogenous progesterone or synthetic progestins such as medroxyprogesterone acetate (MPA) may induce growth hormone (GH) hypersecretion, leading to acromegalic features and insulin resistance [14–16]. This GH excess originates in the mammary gland. Progestins induce the production of GH in foci of hyperplastic ductular epithelium [17,18]. The gene encoding GH in the mammary gland is identical to that in the pituitary gland [19]. Moreover, the progestin-induced mammary-derived protein is identical to pituitary GH and biologically active [17]. The progestin-induced elevations of plasma GH concentrations do not have a pulsatile secretion pattern [20], characteristic of pituitary GH secretion in healthy dogs [21]. Additionally, the progestin-induced GH overproduction can neither be stimulated with GH-releasing hormone (GHRH), nor can it be inhibited by somatostatin, indicating autonomous secretion [20,22].

Locally produced GH probably plays a paracrine role in the progestin-induced proliferation and differentiation of mammary epithelium [19,23]. Because of the similarity of the progestin-induced epithelial changes in both the mammary gland and the uterus, it can be hypothesized that GH is also involved in the development of progestin-induced CEH. Although immunoreactive GH (iGH) has been found in uterine epithelial cells of progestintreated dogs, the absence of mRNA encoding GH in uterine tissue suggests that it does not originate in the uterus [24]. This finding refutes the assumption that local production of GH is involved in the

development of progestin-induced CEH. Therefore, we hypothesized that iGH present in uterine epithelial cells of progestin-treated dogs originates from the mammary gland. To address this hypothesis, the effects of monthly injections with MPA on the development of CEH were investigated in bitches with surgically excised mammary glands and in a control group of bitches with intact mammary glands.

Materials and Methods

Dogs

Thirteen Beagle bitches, 3 to 9 years of age, were housed with outdoor access, fed a commercial dog food once a day, and given water *ad libitum*. The dogs were randomly assigned to two groups (mastectomized and control dogs). The mean age and body weight of the eight mastectomized dogs (5 years and 8 months and 10.5 kg, respectively) did not differ significantly from those of the five control dogs (5 years and 2 months and 9.5 kg, respectively). The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples. Inspection of the vulva (swelling, discharge) was performed and serum progesterone concentration was measured on a regular basis to determine the stage of the estrous cycle.

Surgical procedure

Prior to surgery food was withheld for 18h. After pre-anesthetic medication with acepromazine (Placivet 2 %®, Codifar, Wommelgem, Belgium) and methadone (Mephenon®, Federa S.A., Brussels, Belgium) (iv), anesthesia was induced with propofol (Diprivan 1 %®, Astra Zeneca, Brussels, Belgium) (iv) and maintained with isoflurane (Isoba®Vet, Schering-Plough Animal Health, Middlesex, England) in oxygen. Epidural anesthesia (between lumbar vertebra L7 and sacrum) was performed in dogs that underwent mastectomy and consisted of lidocain hydrochloride (Xylocaïne 2 %®, NV Astra Zeneca, Brussels, Belgium), bupivacain hydrochloride (Marcaïne 0.5 %®, NV Astra Zeneca, Brussels, Belgium), and morphine hydrochloride (Stellorphine®, Stella, Liège, Belgium). Antibiotics (amoxicillin/clavulanic acid (Synulox®, Pfizer Animal Health, Borgo San Michele, Italy)) were administered pre- and postoperatively.

Post surgical analgesia consisted of carprofen (Rimadyl®, Pfizer Animal Health, Dundee, Scotland, UK) and buprenorphine (Temgesic®, Schering-Plough, Brussels, Belgium).

All surgical procedures were performed during anestrous. The tip of the right uterine horn, the corresponding ovary and the entire mammary gland were excised in eight dogs (mastectomized group). Surgical removal of all mammary gland tissue was performed in 2 sessions with an interval of 4-6 weeks. In the five control dogs only the tip of the right uterine horn and the corresponding ovary were excised. At the end of the series of MPA injections, the remainder of the uterus and the left ovary were removed (both dog groups), and a sample of the mammary gland was collected (control dogs). In all dogs intra-uterine fluid was collected for bacteriological examination.

After surgical removal, a part of the tissue samples was fixed in a phosphate-buffered formalin solution, processed, and embedded in paraffin; another part was frozen in liquid nitrogen and stored at -80° C until analysis.

Treatment

Treatment was started in both groups after the first surgical procedures, i.e., during anestrous, and consisted of subcutaneous MPA injections (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) in a dose of 10 mg/kg body weight at intervals of 4 weeks, for a total of 13 administrations.

Blood sample collection and Assays

In all dogs, two blood samples, with an interval of 15 min, for determination of the plasma insulin-like growth factor-I (IGF-I) concentrations were collected by jugular venipuncture before surgery and the first MPA administration, and 1, 4, 7, and 10 months after the onset of treatment.

In all dogs, blood samples for the determination of the plasma profiles of GH were collected at 15-minute intervals between 0800h and 1400h before surgery and the first MPA administration, and 3, 6, 9, and 12 months after the onset of treatment. The blood samples were collected after an overnight fast, immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determinations

Plasma progesterone concentrations were determined with a previously validated radioimmunoassay (RIA) [25]. The intra-assay and interassay coefficients of variation were 7.05 % and 8.75 %, respectively. The sensitivity of the assay was 0.005 ng. Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS, USA). The intra-assay coefficient of variation was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l.

Total plasma IGF-I was measured after acid-ethanol extraction to remove interfering IGF-binding proteins. Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 *g* at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7.4), 13 mM Na_2EDTA, and 0.25 % (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7 %. IGF-I concentrations were measured in a heterologous RIA validated for the dog [26]. The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance CA).

Histology and immunohistochemistry

The formalin fixed, paraffin embedded uterine and mammary tissues were cut at 5 µm for histological and immunohistological staining. For standard histological examination the slides were stained with haematoxylin and eosin (HE). Periodic Acid Shiff (PAS) stainings were made of all uteri with CEH (i.e. after treatment with MPA) and were used to assess the secretory activity of the endometrial glands.

For immunohistochemistry, a polyclonal rabbit anti-porcine GH antibody (generous gift of S.J. Dieleman, Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University) was used in an indirect immunoperoxidase staining procedure (PAP method) as follows. The paraffin wax of the sections was removed with xylene and the sections were cleared in 100 % alcohol. Endogenous peroxidase was blocked by incubation in 1 % H_2O_2 in

methanol for 20 min at room temperature. The sections were rehydrated by passage through 96 % and 70 % alcohol to distilled water. They were then rinsed in phosphate-buffered saline (PBS) (3 x 5 min) and preincubated with normal goat serum in PBS (1:20) for 20 min at room temperature. Incubation with the polyclonal rabbit anti-porcine GH antibody in a 1:5000 dilution in PBS took place overnight at 4° C and then for 30 min at room temperature. After incubation, the sections were rinsed for 3 x 5 min in PBS and incubated with goat anti-rabbit serum (Dakopatts Inc., Glostrup) diluted 1:20 for 60 min. Thereafter, the sections were washed in PBS for 3 x 5 min and incubated with rabbit peroxidase-antiperoxidase complex (Dakopatts Inc., dilution 1:100). Immunoreactive GH was visualized using 0.3 % (v/v) H_2O_2 and 0.5 % (w/v) 3,3,-diaminobenzidine tetrahydrochloride (Sigma, Brussels, Belgium) diluted in 0.05 mol Tris/l in HCl buffer, during a 10 min incubation step. After rinsing in distilled water for 2 min the sections were counterstained with Mayer's haematoxylin for 1 min. A canine pituitary gland served as a positive control. Approximately 70 % of the cells in the adenohypophysis stained positive for GH. The negative controls were sections of uterine and mammary tissue processed in the same way, except that normal rabbit antiserum was used instead of rabbit anti-porcine GH antibody.

Isolation of total RNA and cDNA synthesis

Surgically removed mammary and uterine specimens were snap frozen in liquid nitrogen and stored at -80° C until used for RNA isolation. Total RNA was extracted from 0.2 to 1 g tissue using TRIzol reagent (Invitrogen, Groningen, The Netherlands). Purification was performed with the Rneasy Midi Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's protocol. Purified total RNA was subjected to DNase treatment using the DNA free kit (Qiagen, Leusden, The Netherlands). cDNA synthesis was carried out from 1.5 µg total RNA in 60 µl reaction mix using the iScript^{tm} cDNA synthesis kit (Biorad, Veenendaal, The Netherlands) according to the manufacturer's protocol.

Real time polymerase chain reaction (RT-PCR)

Primers (Table 1) were designed using primer select software of DNA star and primer3 [27] according to the parameters outlined in the Biorad icycler manual. Specificity of each primer pair

was confirmed by sequencing its product. HPRT and GAPDH genes were used as the non-regulated reference genes for normalization of target gene expressions.

RT-PCR was performed using the Biorad MyiQ detection system (Biorad Laboratories Ltd.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 µl containing 12.5 µl of 2 x SYBR green super mixes (Biorad Laboratories Ltd.), 1 µl of each primer at 400 nM concentration, 0.8 µl of cDNA, and 9.7 µl RNase and DNase free water. RT-PCR reactions for each primer set were optimized by performing reactions under a gradient of annealing temperature using five serial 10x dilutions of pooled cDNA from all tissue samples. The protocol used was as follows: denaturation (95° C for 5 min), amplification cycle repeated 40 times (95° C for 30 sec, a PCR specific annealing temperature (Table 1) for 30 sec, 72° C for 30 sec). A melt cure analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were performed in triplicate for every sample. The reference standard dilution series was repeated on every plate. Triplicate negative controls were run with every experimental plate to assess the specificity and to identify any potential contamination.

Table 1. *Primers were designed using primer select software of DNA star and primer3 according to the parameters outlined in the Biorad icycler manual.*

Target gene	Forward primer	Reverse primer	Optimum annealing temperature (°C)
HPRT	agcttgctggtgaaaaggac	ttatagtcaagggcatatcc	56
GH	ctgctgctcatccagtcgt	caggtccttgagcttctcgt	60
GHR	gcgcatcccagagtctaca	accatgacgaaccccatct	58
IGF-I	tgtcctcctcgcatctctt	gtctccgcacacgaactg	60
PR	caatggaagggcagcataac	cagcactttctaaggcgaca	58
GAPDH	tgtccccacccccaatgtatc	ctccgatgcctgcttcactacctt	58

Statistical analysis

The 6-h plasma profiles of GH were analyzed by means of the Pulsar program developed by Merriam and Wachter [28]. The program identifies secretory peaks by height and duration from a smoothed baseline, using the assay SD as a scale factor. The cut-off parameters G1-G5 of the Pulsar program were set at 3.98, 2.40, 1.68, 1.24, and 0.93 times the assay SD as criteria for

accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 5h. The splitting cut-off parameter was set at 0.5 and the weight assigned to peaks was 0.05. The A-, B-, and C- values of the Pulsar program, used to calculate the variance of the assay, were set at A=0, B=7.2, and C=5. The values extracted from the Pulsar analysis included the mean of the smoothed baseline, the pulse frequency, and the area under the curve (AUC). The AUC for GH was calculated above the zero-level (AUC_0) as well as above the baseline (AUC_{base}).

The AUC_0, the basal plasma GH concentration and the plasma IGF-I concentration were analyzed by a mixed model with dog as random effect and time, group (mastectomized and control dog group) and their interaction as categorical fixed effects. Using this model, the mastectomized and control dog group were compared. Additionally the evolution over time of these parameters was evaluated in each group separately. Because the GH pulse frequency and the AUC_{base} were not distributed normally, the two dog groups were compared at each time point (before surgery and MPA injection and 3, 6, 9, and 12 months after the onset of MPA treatment) based on the Mann-Whitney test. Furthermore, the differences within a dog group between 0 months (= before surgery and MPA treatment) and 3, 6, 9, and 12 months after the onset of MPA administration were also analyzed by the Mann-Whitney test. All statistical tests were performed at a global 5 % significance level, applying the Bonferroni correction for multiple comparisons. All values are expressed as mean ± SEM except for the AUC_{base} and the GH pulse frequency which are expressed as median values. Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

The difference in expression of the target genes by RT-PCR between dogs before and after treatment with MPA were assessed using the pair wise fixed reallocation randomization test incorporated in the software program REST-XL [29] at the 5% significance level. Dogs which were subjected to mastectomy before treatment with MPA and dogs with intact mammary gland tissue were treated as separate groups for data analysis.

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Results

In both dog groups, the 6-h plasma profile of GH before surgery and MPA treatment was characterized by a fluctuating baseline with occasional distinct elevations, indicating pulsatile secretion of GH. During treatment with MPA, in the control dogs the basal secretion of GH increased and the pulsatile secretion of GH decreased, whereas in the mastecomized dogs the pulsatile secretion persisted, without changes in the basal GH concentration (Figure 1).

The mean basal plasma GH concentration evolved significantly ($P = 0.0002$) different over time between both dog groups. In the control group the mean basal plasma GH concentration was significantly higher after 9 months (3.4 ± 0.5 µg/l, $P < 0.0001$) and 12 months (2.8 ± 0.6 µg/l, $P = 0.001$) of MPA administration than in the mastectomized group (1.4 ± 0.2 µg/l and 1.6 ± 0.2 µg/l, respectively). Within the mastectomized group the mean basal plasma GH concentration before surgery and treatment with MPA (1.6 ± 0.1 µg/l) did not differ significantly from that after 3 (1.9 ± 0.2 µg/l), 6 (1.1 ± 0.1 µg/l), 9 (1.4 ± 0.2 µg/l), and 12 (1.6 ± 0.2 µg/l) months of MPA treatment. Within the control group the mean basal plasma GH concentration before surgery and treatment with MPA (1.7 ± 0.1 µg/l) was significantly lower than that after 9 months ($P < 0.0001$) and 12 months ($P = 0.0002$) of MPA treatment (Figure 2a).

The mean AUC_0 evolved significantly ($P = 0.0002$) different over time between both dog groups. In the control group the mean AUC_0 after 9 months of MPA treatment (20.4 ± 2.9 µg/lx6h) was significantly higher ($P = 0.0002$) than that in the mastectomized group (9.5 ± 1.5 µg/lx6h). Within the mastectomized group the mean AUC_0 before surgery and treatment with MPA (11.4 ± 0.4 µg/lx6h) did not differ significantly from that after 3 (14.2 ± 1.5 µg/lx6h), 6 (9.8 ± 1.9 µg/lx6h), 9 (9.5 ± 1.5 µg/lx6h), and 12 (11.9 ± 1.3 µg/lx6h) months of MPA treatment. Within the control group the mean AUC_0 before surgery and treatment

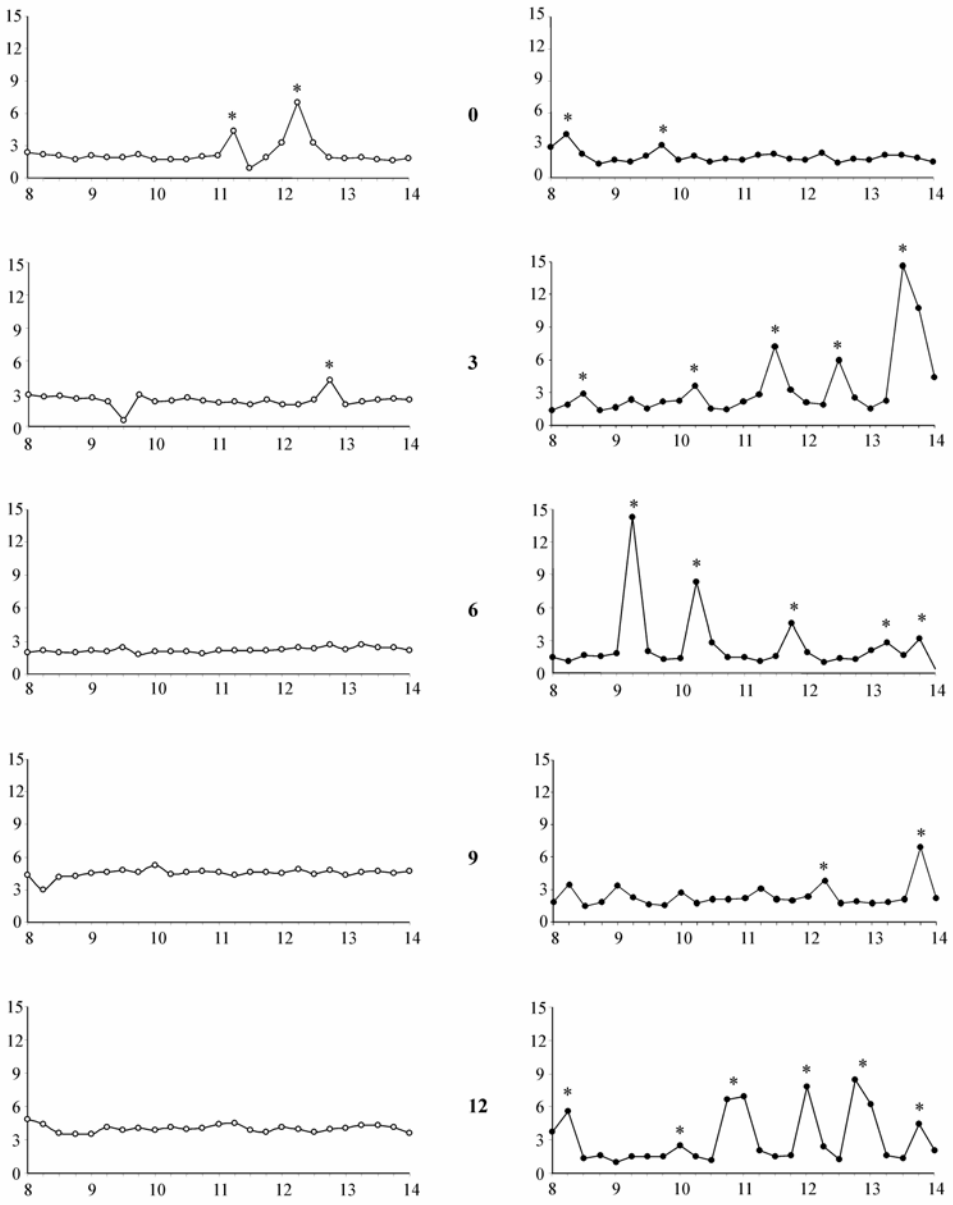

Figure 1. *The secretory profiles of GH (µg/l) (=Y-axis) in a five-year-old mastectomized bitch (●) and in a five-year-old control bitch (○). Blood samples were collected at 15-min intervals for 6 h (from 0800h to 1400h = X-axis), before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment. Significant pulses, calculated by the Pulsar program, are indicated by an asterisk.*

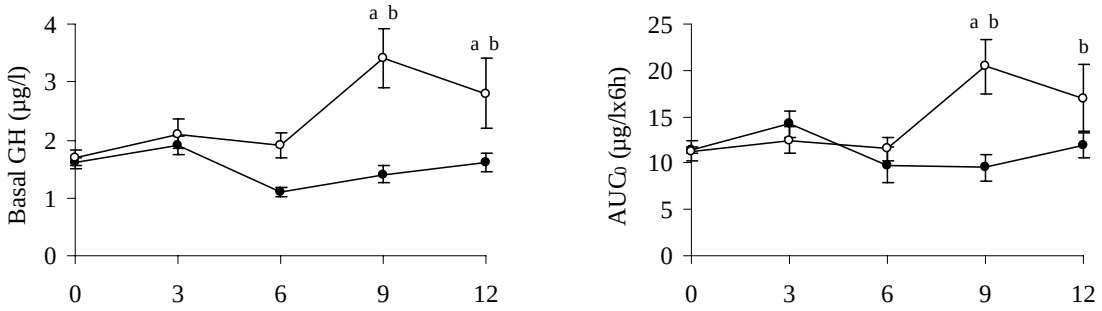

Figure 2. (a) *The mean (± SEM) basal plasma GH concentration,* ***(b)*** *the mean (± SEM) area under the curve for GH above the zero-level (AUC_0) in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○). Blood samples were collected at 15-min intervals for 6 h before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment (= X-axis). 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.*

was significantly lower compared to that after 9 months (20.4 ± 2.9 µg/lx6h, P < 0.0001) and 12 months (17.0 ± 3.6 µg/lx6h, P = 0.005) of MPA treatment (Figure 2b).

The median AUC_{base} in the mastectomized group was significantly higher after 3 months (2.3 µg/lx6h, P = 0.01; range = 0 µg/lx6h to 9.5 µg/lx6h) and 9 months (0.2 µg/lx6h, P = 0.003; range = 0 µg/lx6h to 5.5 µg/lx6h) of MPA administration than in the control group (at both time points median AUC_{base} = 0 and range = 0 to 0). In both groups, the median AUC_{base} before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The median GH pulse frequency in the mastectomized group was significantly higher after 6 months (2 peaks per 6h, P = 0.01; range = 0 to 5 peaks per 6h) and 9 months (1 peak per 6h, P = 0.01; range = 0 to 5 peaks per 6h) of MPA treatment than that in the control group (at both time points median GH pulse frequency = 0 peaks and range = 0 to 0 peaks per 6h).

In both groups the median GH pulse frequency before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The mean plasma IGF-I concentration evolved significantly ($P < 0.0001$) different over time between both dog groups. In the control group, the mean plasma IGF-I concentration was significantly higher after 4 (116 ± 18 µg/l, $P = 0.006$), 7 (143 ± 29 µg/l, $P = 0.001$) and 10 months (180 ± 30 µg/l, $P < 0.0001$) of MPA treatment than in the mastectomized group (54 ± 9 µg/l, 68 ± 12 µg/l, and 57 ± 8 µg/l, respectively). Within the mastectomized group the mean plasma IGF-I concentration before surgery and treatment with MPA (35 ± 7 µg/l) did not differ significantly from that after 1 (35 ± 7 µg/l), 4, 7, and 10 months of MPA administration. Within the control dog group the mean plasma IGF-I concentration before surgery and treatment with MPA (28 ± 6 µg/l) was significantly lower than that after 4, 7, and 10 months of MPA treatment (Figure 3).

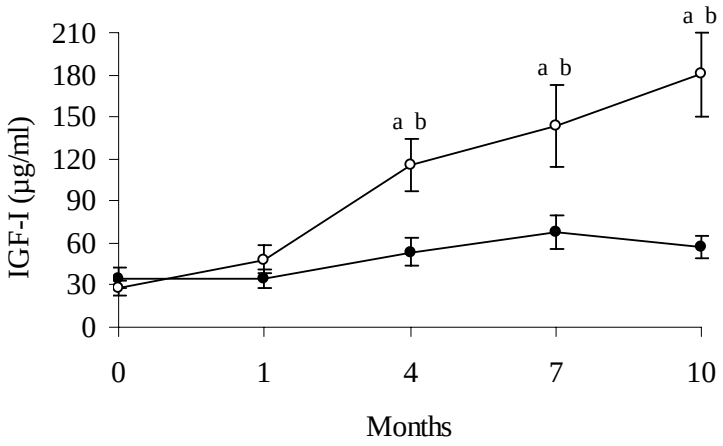

Figure 3. *The mean (± SEM) plasma IGF-I concentration in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○) before surgery and treatment with MPA (0 months) and 1, 4, 7, and 10 months after the onset of MPA treatment. 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.* (*$P < 0.0001$ at all time points)*

Histological examination of the mammary gland tissue before MPA treatment (Figure 4a) revealed inactive mammary tissue characterized by the presence of involuted ductular structures outlined by flattened epithelial cells, absent or small alveolar lumina, and a high stroma/parenchyma ratio. After MPA treatment (Figure 4b), the mammary tissue had differentiated into lobulo-alveolar structures in which milk protein synthesis occurred. The

epithelial cells were cuboidal and surrounded by myoepithelial cells, and the stroma/parenchyma ratio was low. In one dog there was proliferating mammary epithelium, with focal hyperplastic changes of ductular epithelial cells, i.e. ductal budding structures. In the mammary tissues before treatment with MPA, immunohistochemical examination did not reveal iGH. Also after MPA treatment no iGH was found in the mammary tissues, except for the dog with focal hyperplastic changes of ductular epithelial cells (Figure 5a). In this dog, iGH was observed in hyperplastic ductular epithelial cells (Figure 5b).

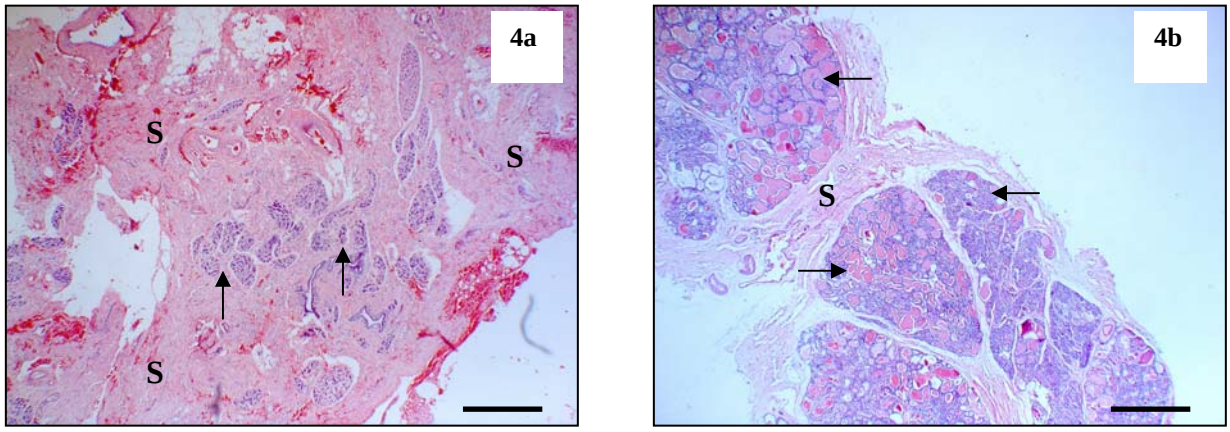

Figure 4. (a) *Histology of mammary gland tissue before MPA treatment revealed inactive mammary tissue characterized by the presence of involuted ductular structures with only remnants of lobules (arrows) and an overrepresentation of stromal cells (S). HE staining - Bar = 200µm.* ***(b)*** *Histology after one year of MPA treatment revealed differentiated lobulo-alveolar glandular mammary tissue with milk protein synthesis (arrows). The amount of stroma is strongly reduced (S). HE staining - Bar = 200 µm*

Histological examination of the uterine tissue before treatment with MPA confirmed that all dogs were in the anestrous phase of the ovarian cycle and that none of the uteri showed histological signs of CEH (Figure 6a). The inactive endometrium contained small endometrial glands outlined by cuboidal cells. The myometrium consisted of smooth muscle cells with dense nuclei and a small amount of cytoplasm. After MPA administration, CEH was macro- and microscopically present in all dogs of both dog groups.

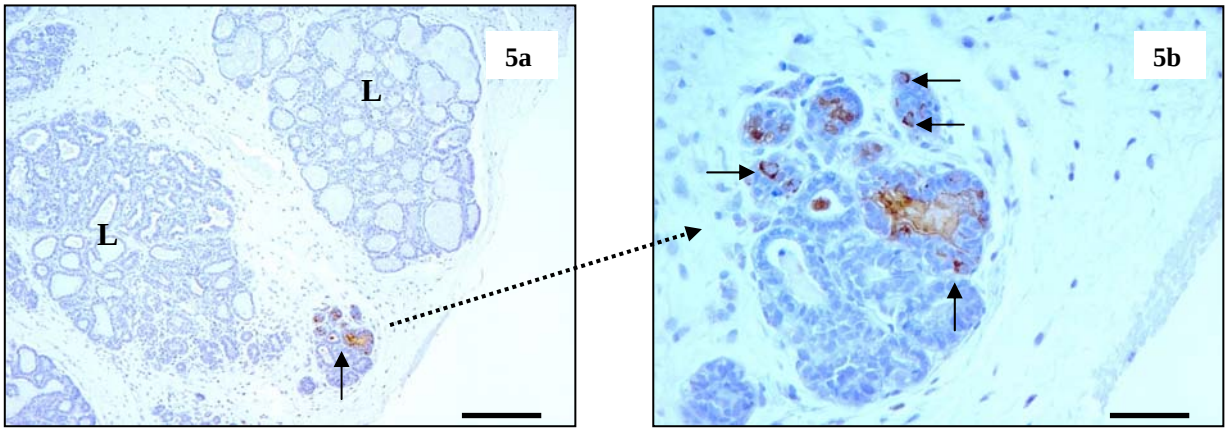

Figure 5. (a) *Immunohistochemical examination of mammary gland tissue in a four-year-old Beagle dog after one year of MPA treatment. Immunoreactive GH is absent in lobulo-alveolar structures (L) and is focally present in hyperplastic ductular epithelial cells ("ductal buds") (arrow). Bar = 200 µm.* ***(b)*** *Enlargement of a part of figure 5a (dotted arrow). Immunohistochemical localization of GH in the cytoplasm of hyperplastic epithelial cells of mammary gland tissue after one year of treatment with MPA (arrows). Bar = 50 µm.*

Histological changes were severe and included multiple, large, mucus-filled cystic, PAS-positive endometrial glands, hyperplasia of the epithelium of the endometrial glands, labyrinth-like proliferations of the surface epithelium and endometrial stroma (Figure 6b). In all dogs, there was a variable degree of necrosis in the endometrium characterized by dense, small nuclei indicating pyknosis, and a small to moderate infiltration with inflammatory cells (neutrophils and foci of lymphocytes and plasma cells). Bacteriological examination of the intra-uterine fluid was negative in all dogs. Immunohistochemical examination of the uterine tissues before MPA treatment did not demonstrate the presence of iGH in any dog. Immunohistochemical examination of the uterine tissues after MPA treatment revealed iGH in all dogs of both dog groups. In general, iGH was located in the cytoplasm of hyperplastic glandular epithelial cells (Figures 7a and b).

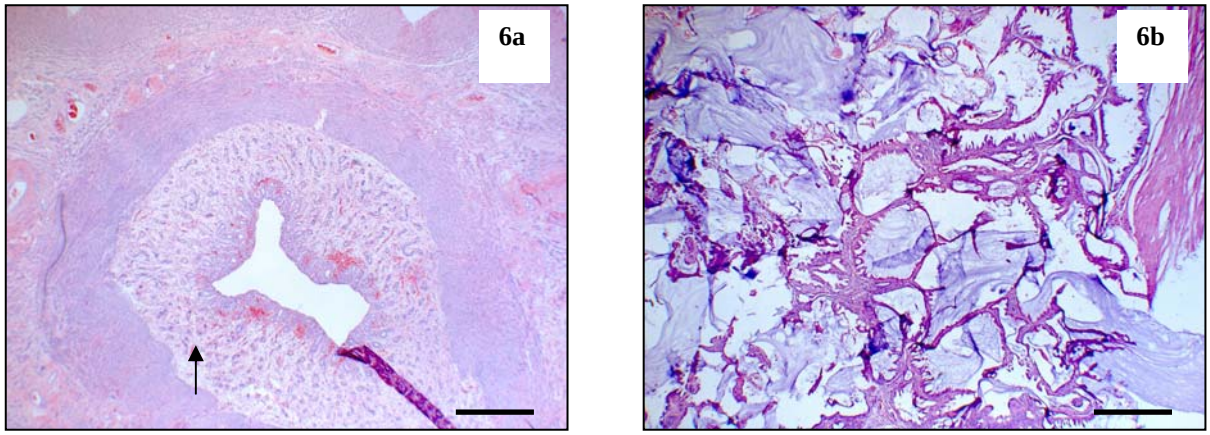

Figure 6. (a) *Histology of the uterus before MPA administration with the characteristics of anestrous, including the endometrium containing small endometrial glands (arrow). HE staining - Bar = 200 µm.* ***(b)*** *Severe CEH after MPA administration characterized by multiple, large mucus-filled endometrial cysts and labyrinth-like proliferations of the surface epithelium. HE staining - Bar = 100 µm*

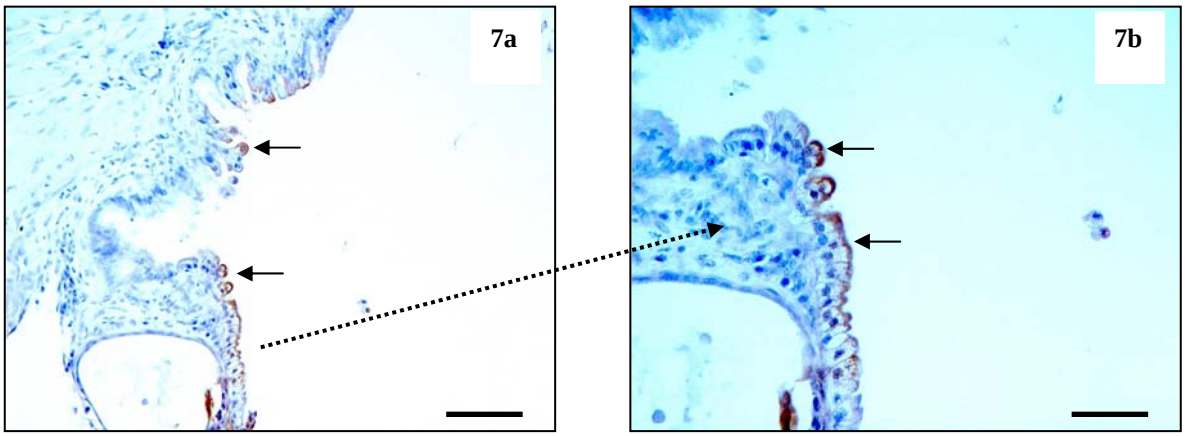

Figure 7. (a) *Immunohistochemical examination of the uterus after one year of MPA administration shows the presence of GH in hyperplastic glandular epithelial cells (arrows). Bar = 200 µm.* ***(b)*** *Enlargement of a part of figure 7a (dotted arrow). Immunoreactive GH in the uterus is present in the vacuolated cytoplasm of the hyperplastic epithelium (arrows). Bar = 50 µm.*

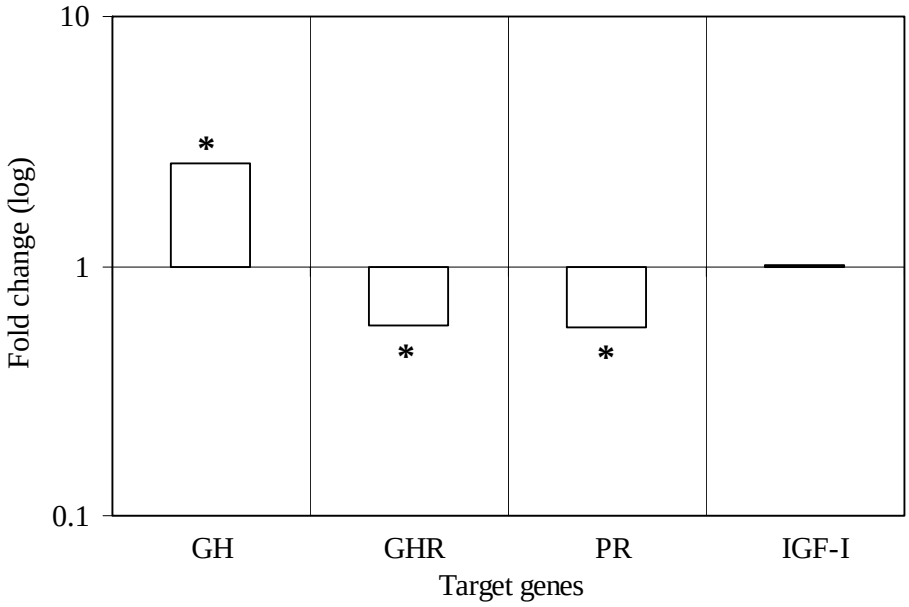

Figure 8. *Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the mammary gland of Beagle dogs demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.*

RT-PCR analysis of the mammary gland tissue of the control dogs after MPA treatment demonstrated a significantly (P = 0.002) higher mean GH gene expression than before treatment. The mean mammary GH receptor (GHR) and progesterone receptor (PR) gene expression was significantly (P = 0.01 and P = 0.03, respectively) lower after MPA administration than before treatment. No significant difference was observed in the mean mammary IGF-I mRNA content after MPA treatment compared to that before treatment (Figure 8). In the mastectomized dogs, the mean GH gene expression in the uterus was significantly (P = 0.002) higher after treatment with MPA than before treatment, whereas in the control dogs, the mean IGF-I gene expression was significantly (P = 0.001) higher after MPA administration. A trend (P = 0.052) for a lower mean GHR gene expression after treatment with MPA was found in the uterus of the control dogs compared with that before MPA administration. In both dog groups the mean mRNA content

encoding the PR after treatment with MPA was significantly (P = 0.001 for both groups) down regulated compared to the PR mRNA content before treatment (Figure 9).

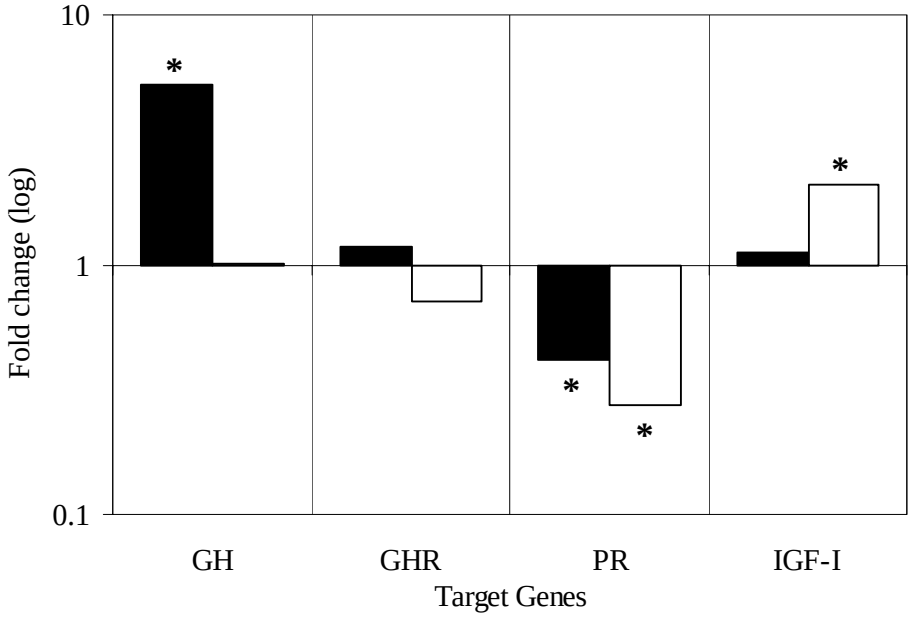

Figure 9. *Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the uterus of five control Beagle dogs (□) and eight mastectomized dogs (■) demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.*

Discussion

In the control dogs MPA treatment resulted in a higher basal GH secretion and less GH secreted in pulses compared to the mastectomized dogs. This is consistent with (partial) suppression of pituitary GH secretion by progestin-induced GH secretion from the mammary gland. In the mastectomized dogs MPA treatment did not result in a change of the basal GH concentration, the AUC_0, the AUC_{base}, and the GH pulse frequency, also demonstrating the mammary origin of progestin-induced GH excess. Dogs are not unique among other mammalian species in that the gene encoding GH is expressed in the mammary gland. RT-PCR has revealed that this gene is

also expressed in the mammary gland of women and cats [19, 30]. However, until now the dog is the only species in which it has been demonstrated unequivocally that progestin-induced mammary-derived GH reaches the systemic circulation, and is biologically active [17].

After several months of MPA treatment, there was a low GH pulse frequency and a corresponding low AUC_{base} for GH in the control dogs compared to the mastectomized dogs. A similarly decreased GH pulsatility has also been reported in women during the second half of pregnancy [31]. In these women the loss of GH pulsatility is due to the release of a placental GH variant [31]. Thus, in both species the loss of GH pulsatility can be ascribed to the negative feedback effects of non-episodically secreted extra-pituitary GH. It has been demonstrated in humans that GH exerts its negative feedback by a stimulation of hypothalamic somatostatin secretion [32]. Additionally, in the control dogs the progestin-induced elevated GH concentrations had induced raised IGF-I concentrations, which will have contributed to the inhibition of pulsatile pituitary GH secretion [33].

With regard to the physiological role of the progestin-induced mammary GH production, local autocrine and paracrine effects in the mammary gland as well as systemic endocrine effects have to be considered. It is thought that the progesterone-induced GH production in the mammary gland leads to local production of IGFs, whereby the growth-promoting effect is modulated by locally synthesized IGF-binding proteins [34]. Thus a proliferative environment for the glandular epithelium is created, i.e., the autocrine/paracrine background for the physiological proliferation and differentiation of mammary gland tissue.

In the dog, the histological features of the mammary gland vary strongly with the stage of the ovarian cycle [35]. In the anestrous phase, the glandular tissue is inactive and microscopically only involuted ductular structures and remnants of lobules are encountered. These were also the characteristics of the mammary gland tissue of our dogs before treatment with MPA. In the luteal phase, after ovulation, there is nodular epithelial proliferation resulting in ductal buds that parallels high endogenous progesterone concentrations in dogs. A similar proliferation of these epithelial cells can be induced by exogenous progestins. In the bitch, ovulation is followed by a relatively long luteal phase, irrespective of pregnancy [36]. During this longstanding progesterone-dominated phase of approximately two months, epithelial cells in budding structures

proliferate and finally differentiate into lobulo-alveolar structures, fully equipped for milk synthesis. In the present study, mammary gland tissue was examined histologically after a long period of progestin administration. This may be the reason that epithelial buds were found in only one dog and that most of the glandular tissue had differentiated into lobulo-alveolar structures in which milk synthesis occurred.

In canine mammary tissue iGH and GH gene expression is found predominantly in the ductal epithelial buds in the early and midluteal phase of the ovarian cycle [18]. In contrast, the GH gene expression is diminished in differentiated lobulo-alveolar glandular tissue and in the anestrous phase of the canine ovarian cycle [18]. Similar immunohistochemical features were found in the present study. iGH was not detected in the mammary gland tissue of the anestrous dogs before treatment with MPA. Additionally, iGH was absent in the mammary gland tissue of the control bitches treated for twelve months with MPA, except for one dog. In this dog, iGH appeared to be present only in hyperplastic ductular epithelium that consisted of more than 2 cell layers, i.e. epithelial cells in budding structures.

RT-PCR analysis, which is more sensitive than immunohistochemical examination demonstrated that MPA administration increased GH gene expression in mammary gland tissue of the control dogs. Also in another study, increased GH mRNA levels were found in mammary gland tissue of dogs after prolonged treatment with progestins [19]. Whereas in primates, GH can bind to the GHR and the prolactin receptor, in nonprimate mammals, such as the dog, GH can only bind to its specific receptor, the GHR [37]. van Garderen et al. [35] demonstrated that immunohistochemical expression of the GHR is down regulated in completely differentiated alveolar epithelial cells at the end of the luteal phase. Similarly, RT-PCR analysis of the mammary tissue in our control dogs after prolonged MPA treatment confirmed that GHR gene expression was significantly lower compared to this expression before MPA administration.

A possible systemic endocrine effect of mammary GH is the effect on uterine epithelium. After MPA treatment the uterine tissues of both the intact and the mastectomized dogs had iGH. This indicates that progestin-induced mammary-derived GH does not play a key role in the development of CEH in the bitch. Nevertheless, the widespread presence of iGH in uterine epithelial cells of dogs with CEH suggests an association between GH and this uterine pathology.

Next to GH, a previous study [38] also demonstrated the presence of iIGF-I in the uterus of dogs with CEH. These growth factors have an intrinsic growth promoting effect on most cell types in the body [39-42]. The growth promoting effect of IGF-I on the uterus is further illustrated by the fact that IGF-I knock out mice have severe hypoplastic uteri [43]. In addition, IGF-I is involved in the postnatal growth of the uterus in the rat [44] and the pig [45]. IGF-I treatment of ovariectomized rats induces uterine endometrial hyperplasia [46].

RT-PCR revealed that, after MPA treatment, the GH mRNA content of uterine tissue was only increased in the mastectomized dogs and not in the control dogs. Comparable with the progestin-induced GH gene expression in canine mammary tissue during development of ductal epithelial buds [18], MPA treatment also resulted in more GH gene expression in the uterine epithelial tissue. Apparently, in the control dogs the elevated circulating concentrations of GH of mammary origin and the subsequently increased plasma IGF-I concentrations suppressed uterine GH gene expression, similar to what has been reported for the pituitary [33]. MPA treatment also resulted in increased expression of the IGF-I gene in the uterine tissue, but this was significant only in the control dogs. This may be explained by the stimulating effect of the elevated circulating concentrations of GH, originating from the mammary gland, on uterine IGF-I gene expression in these dogs. As the presence of mRNA encoding for the GHR has already been demonstrated in the human uterus [47,48], circulating GH may indeed influence intracellular effects in uterine cells.

In this study MPA did not promote the expression of GHRs in uterine epithelium. This makes it unlikely that increased numbers of GHRs can explain the presence of iGH in uterine cells, as proposed earlier [24].

Uterine steroid hormone receptor status is a crucial element in pathological conditions such as CEH and pyometra [8,49]. Progesterone exposure is considered the initiating step in the development of CEH [10-13,50-52]. In the present study one year of MPA treatment decreased the expression of the gene encoding for PR. In line with this observation, immunohistochemical studies [53] found a severe reduction in the canine uterine PR after 12 weeks of MPA administration. The uterine concentration and distribution of progesterone receptors are influenced by hormonal changes during the estrous cycle and by administration of MPA in the

bitch [54]. An increase in plasma progesterone concentration results in a decline of PR content in the uterus [55-59] and explains the decrease of PR mRNA with MPA treatment in the present study.

In conclusion, MPA administration caused hypersecretion of GH and IGF-I in the control dogs, but failed to do so in the mastectomized dogs. All dogs of both groups developed CEH, indicating that mammary GH is not a requirement for the development of progestin-induced CEH. Nevertheless, the presence of iGH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.

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Chapter 6

Enhanced Wnt signaling in progestin-induced canine mammary hyperplasia and spontaneous mammary tumors

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(Submitted)

Abstract

Progesterone plays an important role during normal development and tumorigenesis of mammary gland. The downstream effectors of progesterone signaling are not clearly understood. Progestins induce mammary gland hyperplasia and a mammary-derived growth hormone (GH) excess in female dogs. Wnt4, a ligand of canonical Wnt signaling was identified as a target gene of progesterone in developing mouse mammary gland. Spontaneous mammary tumors of women as well as of female dogs often display stabilized β-catenin immunoreactivity and overexpression of CyclinD1, a hallmark of aberrant activation of canonical Wnt signaling. In order to gain further insight in to putative progesterone-Wnt pathway crosstalk in mammary tumorigenesis and progression, we investigated the Wnt pathway activity in progestin-induced canine mammary hyperplasia (CMH), canine mammary carcinomas (CMC) and tumor cell lines (CMT). Progestin-induced CMH and spontaneous CMC showed amplified expression of Wnt ligands, target genes and increased stabilized β-catenin immumoreactivity compared to normal mammary tissue indicating an activated Wnt signaling. Out of three well-established CMTs, one showed constitutively active Wnt signaling and also responsiveness to Wnt pathway modulation. Treatment of this cell line with the previously identified small molecule inhibitor of canonical Wnt signaling was associated with a dose-dependent inhibition of Wnt target gene expression and a tendency to decreased TCF reporter activity. It is concluded that, progestin-induced canine mammary hyperplasia is associated with overexpression of Wnt ligands and subsequent activation of the pathway. Its relation to GH signaling and possible therapeutic interventions warrants further research .

Introduction

Breast cancer is the most common cancer of women [1, 2]. In addition to many other factors contributing to its incidence and progression, breast cancer is clearly a hormone-dependent malignancy. Ovarian hormones, mainly estrogen and progesterone have been implicated in mammary tumorigenesis in women [3]. A women's health initiative (WHI) study reported that hormone replacement therapy with a combination of a progestin and estrogen increased the risk of developing a breast cancer compared to estrogens alone indicating a strong oncogenic potential of progesterone [4]. Domestic animals as well as wild zoo animals treated with the synthetic progestins are known to develop mammary cancer, which further endorses the conserved oncogenic potential of progestins (reviewed in [5, 6]). Animal models have proved to be very useful in understanding the mechanism of progestin action in the mammary gland. As a result, it is proposed that progesterone regulates the local production of growth factors in target tissues, which in turn in an autocrine/paracrine manner regulate cell proliferation [7]. This is in agreement with our findings in dogs, which have shown that progestins induce a local, mammary gland specific expression of growth hormone (GH) [6, 8, 9]. Based on studies using a mouse model the Wnt signaling ligand Wnt4 has also been suggested as one of the candidate growth factors mediating the effect of progestins on mammary epithelial cells [10]. These studies indicate the possible involvement of multiple downstream signaling cascades in delivering mitogenic effect of progesterone to the target tissue.

The Wnt signaling plays a critical role in embryonic development, adult tissue maintenance, cell fate decisions and is also implicated in tumorigenesis in several tissues [11, 12]. Wnt signaling is activated by a family of secreted Wnt proteins that bind to the transmembrane co-receptor complex containing frizzled proteins (Fz) and LDL receptor related proteins (LRP5 or LRP6) [13, 14]. Wnt proteins are believed to signal through three distinct pathways: the canonical or Wnt-β-catenin pathway, the Wnt-Ca^{2+} pathway and a planar cell polarity pathway [15]. Of these pathways, the canonical signaling is well characterized and is known for its role in tumorigenesis in various tissues, including the mammary gland. The key molecule in canonical Wnt signaling is β-catenin (reviewed in [12]). In a normal non-stimulated cell, the majority of β-

catenin is bound to E-cadherin, a transmembrane protein involved in cell adhesion [16]. Remaining β-catenin in the cytoplasm is phosphorylated by the β-catenin destruction complex consisting of Axin, adenomatous polyposis coli (APC), casein kinase (CK1 or CK2) and glycogen synthase kinase 3 beta (GSK-3β) which is subsequently degraded by the ubiquitination complex [17, 18]. Bound by their ligands, the Fz/LRP co-receptor complex activates the canonical Wnt signaling pathway by dissociating the β-catenin destruction complex [11]. As a consequence, stabilized β-catenin translocates to the nucleus and regulates target gene transcription by associating with T-cell factor/lymphocyte enhancer factor 1 (TCF/LEF1) transcription factors [19-21]. TCF/LEF1 transcription factors activate many target genes including CyclinD1, c-myc, Axin2 and Survivin [22-25]. The oncogenic potential of Wnt signaling is dependent on functions of its target genes, which extend from cell proliferation, inhibition of apoptosis to cell motility and invasion.

Hyperactivation of the canonical Wnt signaling due to mutations in its key components such as β-catenin, APC and Axin is frequently observed in various human cancers [26-28]. In breast cancer, however, evidences of comparable mutations are lacking. Nevertheless, there is strong evidence based on immunohistochemistry that more than 50% of breast carcinomas display stabilized β-catenin, an indicator of the active canonical Wnt signaling [29, 30]. Possible explanations for stabilized β-catenin are stimulation by other deregulated signaling pathways (EGFR/P53) [31, 32], mutations in other components of the pathway that have not been systematically scrutinized and overexpression of Wnt ligands. Recently, overexpression of Wnt proteins has been reported in human breast cancer and cell lines [33, 34].

Mammary tumors of dogs are very similar to those of humans in many aspects, such as being spontaneous and clearly hormone dependent. The tumor incidence in non-spayed dogs is even more frequent than in women [35, 36]. The higher frequency of canine mammary tumors could be related to the prolonged exposure to progesterone during the canine estrus cycle. To further support this hypothesis, prolonged progestin treatment in ovariectomized beagle dogs resulted in mammary hyperplasia and benign tumors [8, 37, 38]. In addition to its sensitivity to progestins, a high level of genetic diversity makes dog a unique species to study the mechanism of progesterone mediated mammary tumorigenesis.

Increasing evidences of active canonical Wnt signaling in mammary tumors stresses the importance of further investigations towards the driving force behind this activation. In this study, we investigated the activity of canonical Wnt signaling in progesterone induced canine mammary hyperplasia (CMH) and spontaneous carcinomas (CMC). In addition, the activity of Wnt signaling was also quantified in canine mammary tumor cell lines (CMT) and the effect of a putative inhibitor of TCF and β-catenin interaction [39, 40] was evaluated.

Material and methods

Canine mammary tissue

Mammary tissue from eight control and eight progestin-treated dogs was obtained from our tissue bank originating from previous studies [8, 9]. Detailed animal experiment protocols were reported earlier. Canine mammary carcinomas were routinely collected from the patients that have been referred to the Utrecht University Clinic for Companion Animals, snap frozen using liquid nitrogen and stored at -80^{0}C or fixed and paraffin embedded.

RNA isolation and Real time quantitative-PCR (qRT-PCR)

Total RNA from canine mammary carcinomas and cell lines was isolated using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Synthesis of cDNA was carried out from 1.5 µg total RNA in 60 µl reaction volumes using the iScript^{TM} cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, The Netherlands). Primers (Table 1) were designed using primer select software of DNA star (Madison, WI) according to the parameters outlined in the Bio-Rad i-cycler manual. The specificity of each primer pair was confirmed by sequencing its product. RPS19 and HPRT genes were used as the non-regulated reference genes for normalization of target gene expression [41]. qRT-PCR was performed using Bio-Rad MyIQ detection system (Bio-Rad) with SYBR green fluorophore. Data analysis was carried out using the pairwise fixed reallocation and randomization test incorporated in the software program REST-XL [42] at 5% level of significance. Every experiment was performed in triplicate and the average values from 4-5 independent experiments were used for data analysis.

Table 1. *Primer pairs used for quantitative RT-qPCR; U- forward primer, L = reverse primer, T = annealing temperature.*

Gene name	Primer orientation	Primer sequences(5'-3')	T (^{0}C)
Wnt-2	U	GACAGGGATCACAGCCTCTT	63
	L	TGGTGATGGCAAACACAACT	
Wnt-3	U	ATGAACAAGCACAACAACGAG	61.5
	L	TTGAGGAAGTCGCCGATAG	
Wnt-4	U	CGAGGAGTGCCAGTACCAGT	61
	L	CGAGGAGTGCCAGTACCAGT	
Wnt-5a	U	TGCCACTTGTATCAGGACCA	61
	L	GCTGCCTATCTGCATGACC	
Wnt-5b	U	GCGGAGGGCTGTGTATAAGA	58
	L	GTCCCCTACTTTGCGGAACT	
Wnt-7a	U	GCCTCGACGAGTGTCAGTTT	60
	L	GATGATGGCGTAGGTGAAGG	
Wnt-10b	U	TTCTCTCGGGATTTCTTGGA	60
	L	CATTTCCGCTTCAGGTTTTC	
CyclinD1	U	GCCTCGAAGATGAAGGAGAC	60
	L	CAGTTTGTTCACCAGGAGCA	
Survivin	U	CCTGGCAGCTCTACCTCAAG	58
	L	TCAGTGGGACAGTGGATGAA	
Axin2	U	CACCCGCTCTACAACAAGGT	60
	L	AGGTGGAGATGAAGCACAGC	
c-myc	U	GCCGGCGCCCAGTGAGGATA	61
	L	GCGAACGCAACATAGGAGGGAGAGC	
β-catenin	U	ATGGGTAGGGCAAATCAGTAAGAGGT	64
	L	AAGCATCGTATCACAGCAGGTTAC	
RPS19	U	CCTTCCTCAAAAAGTCTGGG	61
	L	GTTCTCATCGTAGGGAGCAAG	
HPRT	U	AGCTTGCTGGTGAAAAGGAC	56
	L	TTATAGTCAAGGGCATATCC	

Cell culture

The experiments were performed using three canine mammary cell lines CMT-U229, CMT-U335 and P114. Detailed characterization of these cell lines was reported earlier [43-45]. The cells were grown in DMEM-F12 (Invitrogen, The Netherlands) supplemented with 10% foetal calf serum (FCS) (Harlan Sera-Lab, UK) and 1% Penicillin/streptomycin (Invitrogen) at 37^{o}C/5% CO_2. In general, cells were passed upon reaching 70-80% confluence. For counting, cells were stained with 0.05% Trypan blue in saline (Seromed-Biochrom KG, Germany). Cercosporin (identical to CGP049090), LiCl and pyridoxine were purchased from Sigma-Aldrich (Sigma-Aldrich, The Netherlands).

Cell viability assay

Cell viability was estimated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay as described previously with minor modification to the original protocol [46]. In brief, cell lines were seeded in 96-well plates (Becton Dickenson, The Netherlands) at density of 1500 - 3000 cells/well. Using 40μM pyridoxine, a known singlet oxygen quencher, cercosporin treatment conditions were optimized in order to eliminate its light dependent toxicity. After 24 h, cells were treated with culture medium containing 0.5-8 μM of cercosporin alone or in combination with 5 mM LiCl for 54 h. Control cells were treated with cell culture medium containing equivalent concentration of carrier, absolute ethanol. After 54 h, cells were incubated with MTT (0.45 mg/ml of final concentration) for 1.5 h. Next, the cell culture medium with MTT was removed, cells were lysed with acidic isopropanol (100 μl/well) and the absorbance of the dissolved formazan was measured at 595 nm with 650 nm as reference wavelength using a microplate reader (BioRad, Model 3550). Cell number for every time point was deduced from absorbance using the standard curve (serial dilution of cells) for each cell line. In a parallel experimental setup, cell lines were treated with cercosporin, LiCl or both in combination for gene expression studies. Experiments were repeated 3-4 times.

Transient transfections and TCF-reporter assay

Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, The Netherlands) in 24-well plates (BD Biosciences, The Netherlands). For that purpose, cell lines

were seeded 48 h before transfection at density ranging between 38000-66000 cells/well. The cells were transfected with firefly luciferase reporter plasmids, pTOPFLASH and pFOPFLASH (gift from prof. H. Clevers, Hubrecht Institute, Utrecht, The Netherlands). Transfection conditions for individual cell lines were optimized. CMT-U229 and CMT-U335 were incubated with 0.8 µg reporter plasmid and 3 µl lipofectamine, while for P114 1.2 µg plasmid and 4 µl lipofectamine was used. Transfection was carried out by adding to each well 500 µl of serum-free DMEM (Sigma-Aldrich), pTOPFLASH/pFOPFLASH reporter plasmid, lipofectamine and 100 ng of the renilla luciferase reporter (Promega, The Netherlands) as an internal control. Cells were incubated for 5 h followed by replacement of transfection medium with the cell culture medium. 24 h after transfection cells were treated with LiCl or cercosporin for 16-20 h. Cell lysis was performed using 100 µl of passive cell lysis buffer, and the luciferase activity was determined with a luminometer (Centro LB 960; Berthold technologies, Belgium) using a Dual Luciferase Assay System (Promega) on 25 µl of lysate.

Immunohistochemistry

Formalin fixed and paraffin embedded tissue samples (normal, CMH and CMC) were cut in to 5 µM sections and placed on poly-l-lysine coated slides (Sigma, The Netherlands), deparafinized and re-hydrated. Immunohistochemical staining was performed using avidin-biotin-peroxidase method. For antigen retrieval, sections were immersed in 10 mM citrate buffer (pH 6.0) and boiled in microwave at 1000 W for 2.5 min. Sections were dehydrated and endogenous peroxidase activity was blocked by incubation with 1% H_2O_2 in 100% methanol for 30 min. Then, sections were re-hydrated again and washed three times in PBS. Non-specific antibody binding was blocked by pre-incubation with 3% BSA solution containing 0.025% (v/v) TritonX in PBS for 30 min at room temperature. Rabbit anti-human β-catenin polyclonal antibody (AB2982; Abcam, UK) which is reported to recognize canine β-catenin was used as primary antibody. Sections were incubated with primary antibody (dilution 50x) for 90 min at room temperature. Next slides were rinsed 2 times (5 min each) in PBS + 0.025% (v/v) Triton X-100 and incubated in biotinylated goat anti rabbit secondary antibody (K4004; Dako, Denmark). Slides were washed 2 times (5 min each) in PBS and dried. Next, slides were incubated in 200 µl DAB (Sigma)

solution for 5 min followed by a brief rinse in water. Sections were counterstained with Mayer's hematoxylin for 1-2 seconds, dehydrated in ethanol and xylene, and mounted with glass cover slips using Eukitt. All images were acquired using Leica DM RE light microscope (Leica Microsystems, Germany) at 20x magnification.

Statistical analysis

Mean values from 4-6 independent experiments of treatment and control were compared for significant difference using student's t-test and expressed as percentage of control values. Similarly, statistical significance of difference in gene expression between normal tissues and CMHs or CMCs was estimated using students t-test.

Results

Expression of Wnt ligands and target genes in progestin-induced mammary hyperplasia

The effect of progestin administration on the expression of Wnt ligands in canine mammary tissue was analyzed using mammary tissue from healthy control dogs and dogs after prolonged treatment with progestins [8]. The concentrations of mRNA encoding Wnt2, Wnt3, Wnt4, Wnt5a, Wnt5b, Wnt7a and Wnt10b genes were quantified by real time RT-PCR. Transcripts of all examined Wnt ligands were detectable in canine mammary tissues. Mean mRNA concentrations of Wnt4, Wnt3 and Wnt5a were significantly higher (115, 2 and 1.6 fold, respectively) and Wnt2 levels were 2.3 fold lower in CMH when compared to the normal tissue (Fig 1a). Wnt5b, Wnt7a and Wnt10b expression was not significantly different between the two groups. As Wnt3 and Wnt4 are known to activate canonical Wnt signaling, we tested the functional relevance of their overexpression in terms of β-catenin stabilization and canonical Wnt target gene induction. Expression of β-catenin mRNA did not differ between the two groups (Fig 1b), but immunohistochemical analysis revealed higher levels of stabilized β-catenin protein in CMH compared to normal tissue (Fig 2). In addition, CMH also showed significant elevation of CyclinD1, Survivin, Axin2 and c-myc mRNA concentrations (2, 4, 1.8, and 1.4 fold increase, respectively) compared to normal tissue (Fig1b).

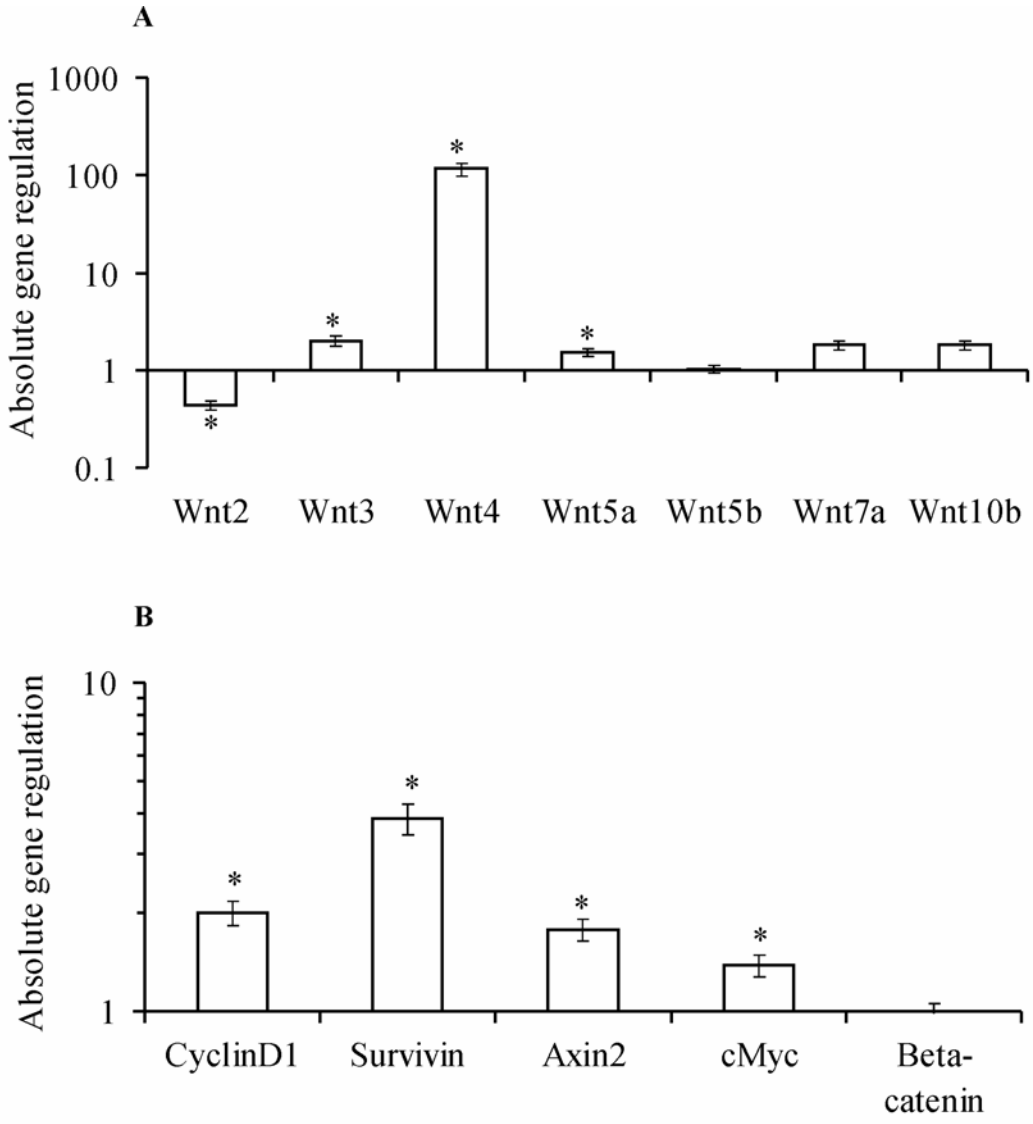

Figure 1. *RT-qPCR quantification of Wnt ligands* ***(A)****, β-catenin and Wnt signaling target gene expression* ***(B)*** *in progesterone induced CMH. The values are expressed as the ratio to that of non-treated mammary tissues (±SEM).* ****= p≤0.05*** *compared to non-treated normal mammary tissue.*

Overexpression of Wnt ligands in canine mammary carcinomas (CMCs)

Next expression of ligands and target genes of the canonical Wnt pathway were examined in spontaneous carcinomas (CMC). Known canonical Wnt target genes, namely, CyclinD1, Axin2, Survivin and c-myc were upregulated by, 2, 2, 2.5, and 1.5 fold, respectively in CMH compared

to healthy control tissue. However, the pattern of Wnt mRNA concentrations were different from that after progestin treatment with significant higher concentrations of Wnt7a, Wnt4, Wnt3, Wnt5a and Wnt5b mRNA of 6.8, 3, 2.8, 2 and 2.2 fold, respectively ($p<0.001$) in CMCs. Expression of Wnt2 and Wnt10b was not significantly different between the two groups (Fig 3). Increased immunoreactivity of stabilized β-catenin is also evident in CMCs (Fig 2).

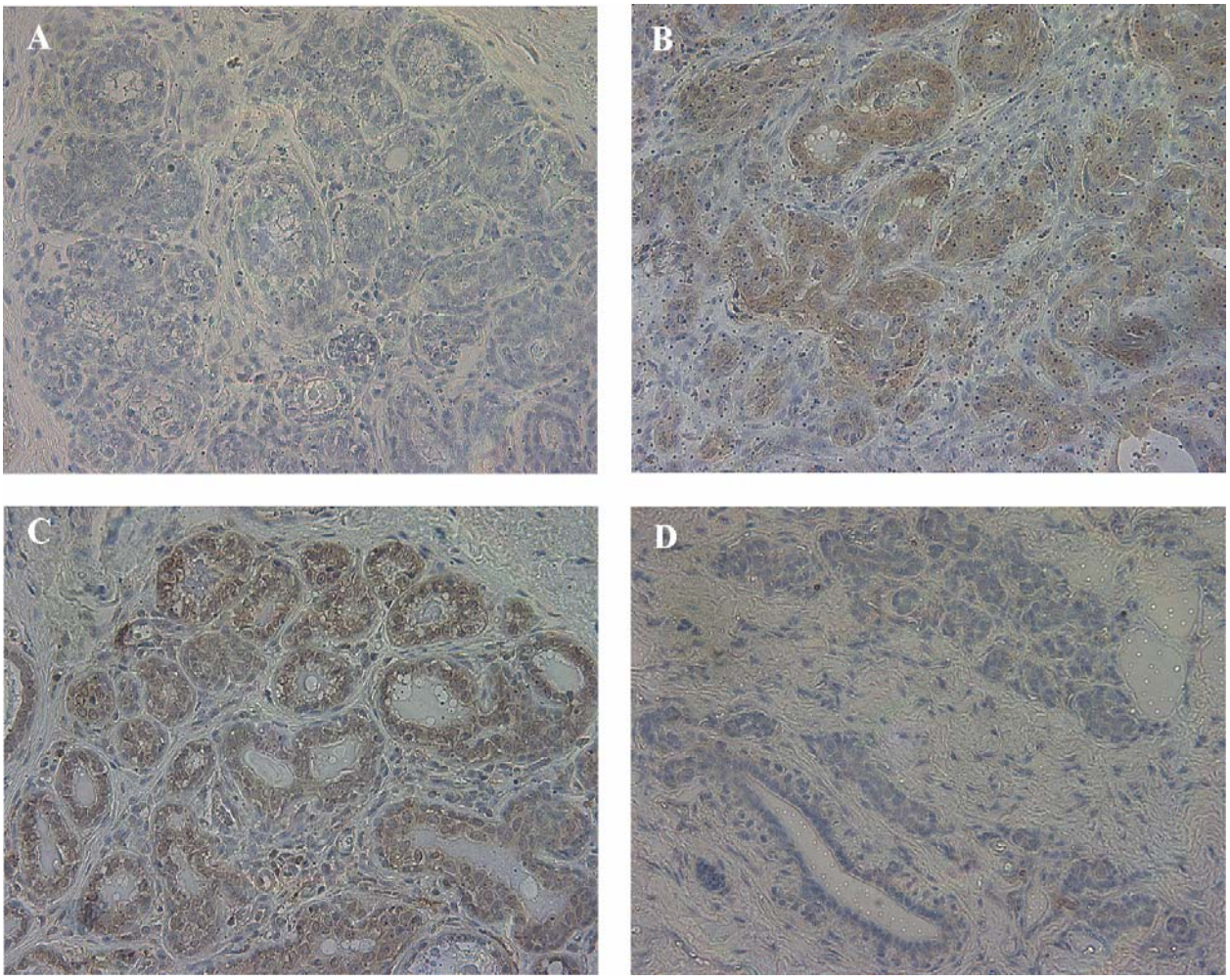

Figure 2. *Immunohistochemical analysis of canine mammary tissue using rabbit anti-human-β-catenin antibody;* ***(A)*** *negative control (CMH incubated with rabbit serum)* ***(B)*** *canine mammary tumor* ***(C)*** *canine mammary hyperplasia (CMH) and* ***(D)*** *normal mammary gland. All images are taken at 20x magnification.*

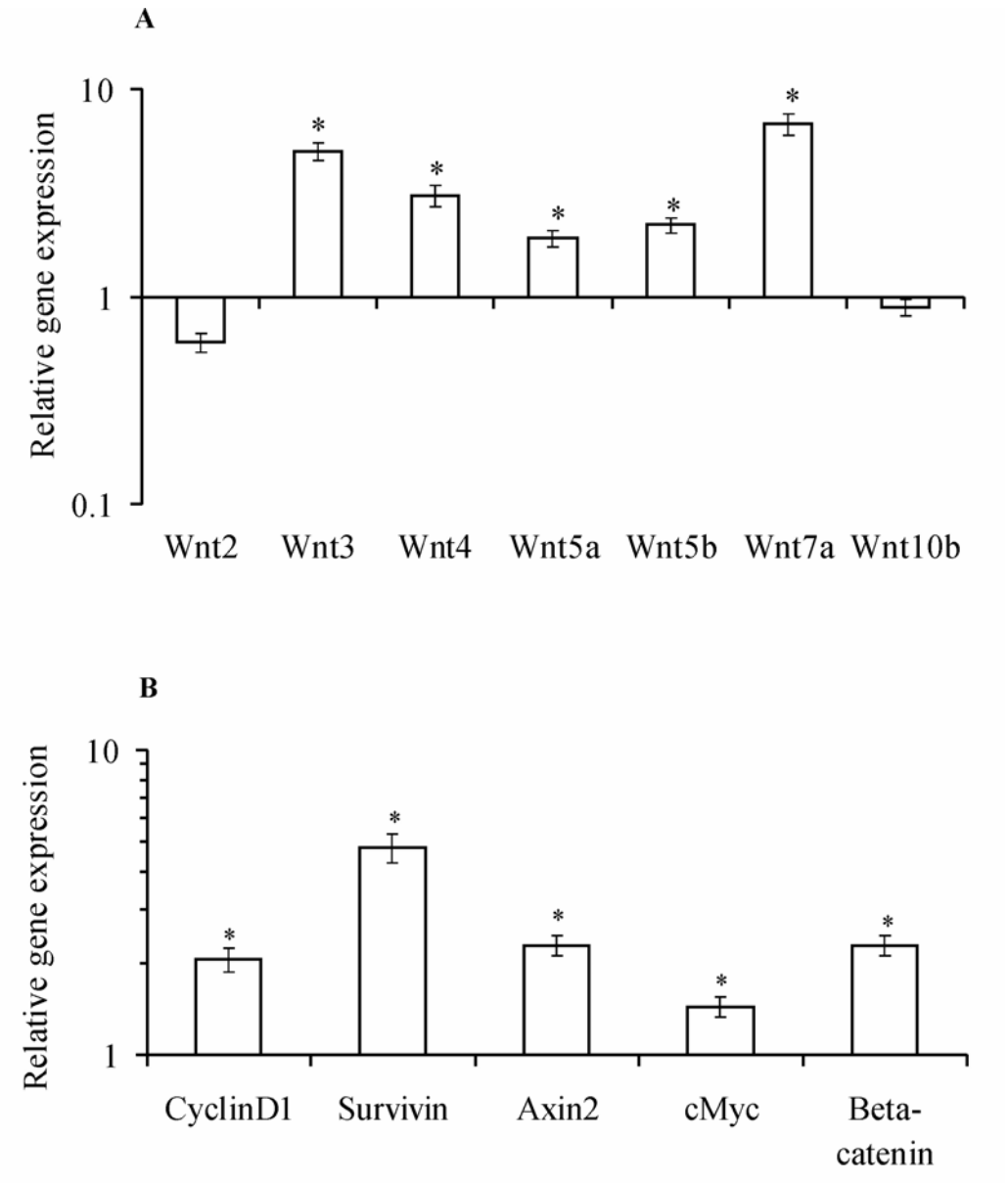

Figure 3. *RT-qPCR quantification of Wnt ligands* ***(A)****, β-catenin and Wnt signaling target gene expression* ***(B)*** *in canine mammary carcinomas. The values are expressed as the ratio to that of normal mammary tissue (±SEM).* ****=*** *$p \leq 0.05$ compared to of normal mammary tissue*

Constitutive Wnt signaling in canine mammary cell line (CMT*)*

Three well described canine mammary cell lines were used to investigate Wnt signaling in vitro. A TCF reporter construct pTOPFLASH (TOP) or a reporter plasmid pFOPFLASH containing

mutated TCF-binding sites (FOP) were transfected separately. Differences in transfection efficiency were corrected by simultaneous transfection of a renilla reporter. In cell lines CMT-U229 and CMT-U335 a TOP/FOP ratio close to 1 indicated negligible basal β-catenin/TCF activity whereas cell line P114 demonstrated a high (TOP/FOP ratio of 4.5) basal canonical Wnt pathway activity (Fig 4a). Treatment with the GSK3β inhibitor LiCl resulted in a 3-fold increase in TOP/FOP ratio in cell line P114 and CMT-U229 while CMT-U335 responded with moderate

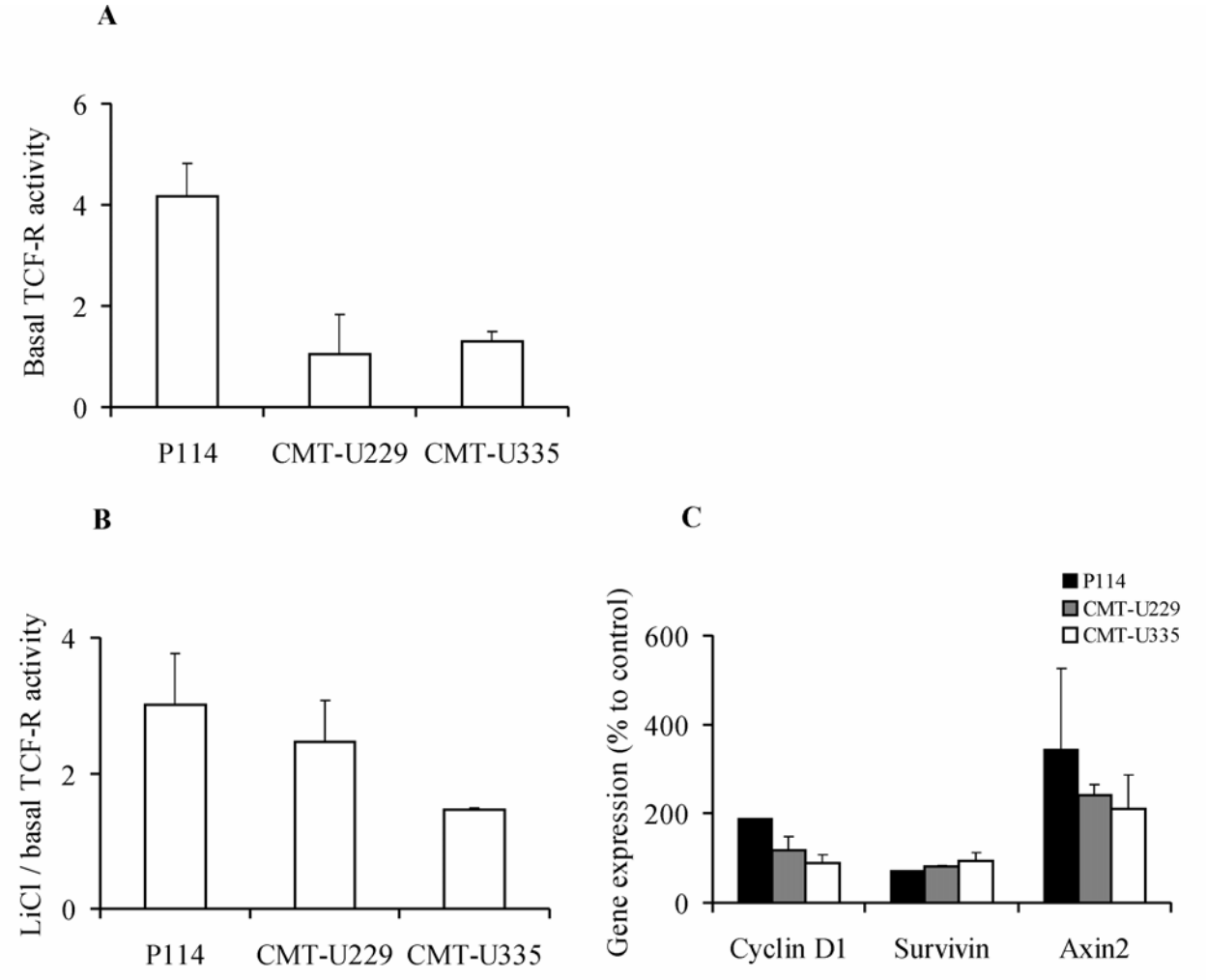

Figure 4. *TCF/Luciferase (TOP) assay quantifying the activity of β-catenin/TCF pathway in canine mammary tumor cell lines* ***(A)****. LiCl stimulation of TCF-reporter activity expressed as the ratio to the basal activity (non-treated)* ***(B)****. qRT-PCR quantification of canonical Wnt target gene expression in CMTs 18 h after 5 mM LiCl treatment* ***(C)****.*

increase (Fig 4b). Treatment with LiCl was associated with increased expression of the canonical Wnt target genes CyclinD1 and Axin2 but no stimulatory effect on Survivin expression. Cell line P114 showed the highest induction of the target gene expression, compared to CMT-U229 and CMT-U335 (Fig 4c).

Wnt pathway inhibition using proposed small-molecule inhibitor CGP049090 (cercosporin)

In a recent study, a library of natural compounds was screened for potent small molecule inhibitors of β-catenin/TCF interaction. CGP049090 was identified as a candidate compound [40] and in a structure based search in NCI database identified as the commercially available fungal toxin cercosporin. As cercosporin is known for its light-dependent production of singlet oxygen experiments were conducted in the dark and the absence or presence of the singlet oxygen quencher, pyrodoxine. Cercosporin treatment resulted in a dose-dependent decrease in cell viability and a greater sensitivity of cell line P114 (Fig 5). Pyridoxine did not prevent the decrease in viability indicating that the observed effects are not due to production of the singlet oxygen (data not shown).

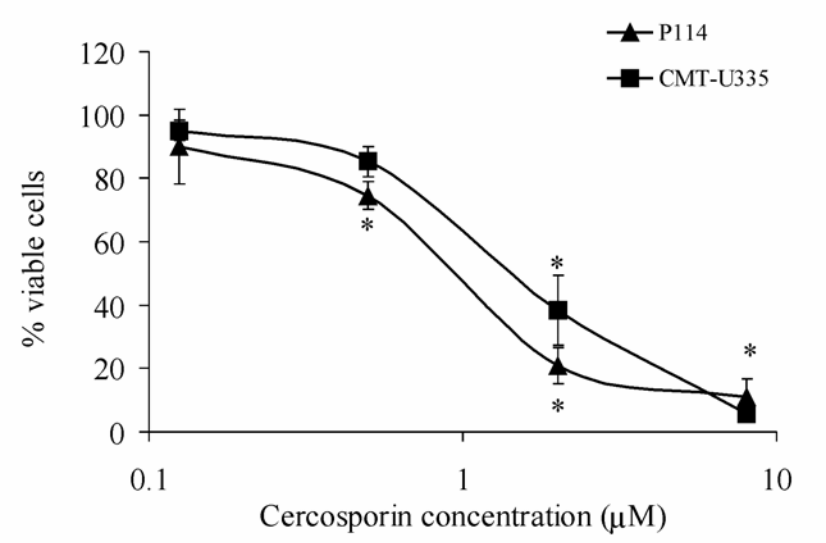

Figure 5. *Effect of cercosporin on cell viability of canine mammary cell lines estimated using a MTT-assay.*

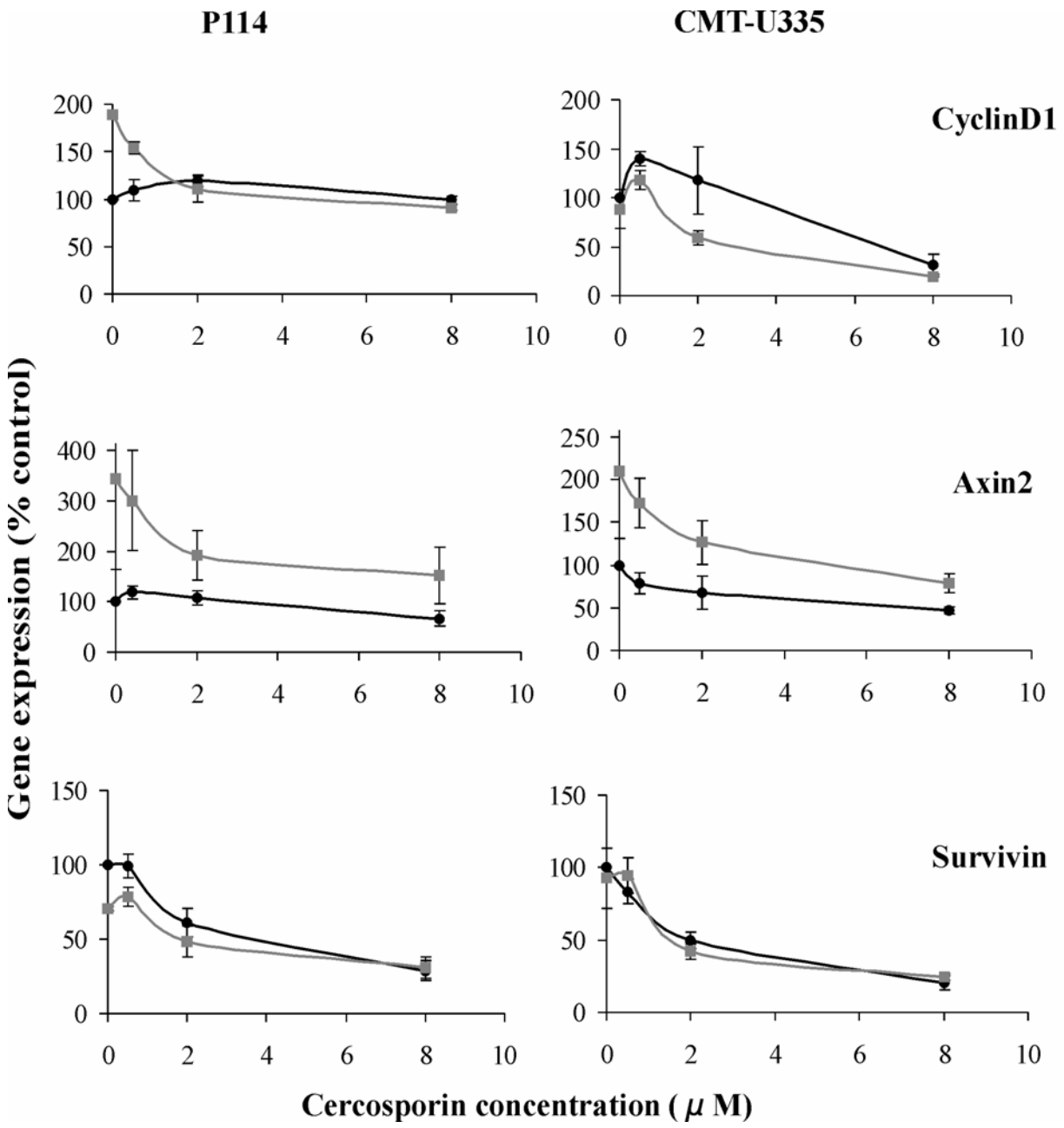

Figure 6. *Cercosporin mediated suppression of canonical Wnt target gene expression in canine mammary cell lines. Cells were treated with cercosporin alone are in combination with the known Wnt pathway activator LiCl. Gene expression was quantified using qRT-PCR and the normalized mean values of 3-4 independent experiments were expressed as the ratio to that of non-treated cells. Legends;* ■ *cercosporin,* ● *cercosporin + 5 mM LiCl*

Cercosporin treatment resulted also in a dose dependent inhibition of the expression of CyclinD1, Axin2 and Survivin mRNA in CMT-U335, and of Axin2 and Survivin mRNA in P114. Although no significant inhibition of CyclinD1 mRNA was found in P114, cercosporin dose dependently attenuated the LiCl stimulation of CyclinD1 expression in this cell line (Fig 6).

Cercosporin clearly inhibited the TOP-reporter activity, dose dependently with IC50 being approximately 8 μM (Fig 7a), but also inhibited the FOP-reporter activity resulting in only a tendency to a decreased TOP/FOP ratio. In CMT-U335, cercosporin did not affect FOP-reporter activity with only a small decrease in TOP-reporter activity (Fig 7b).

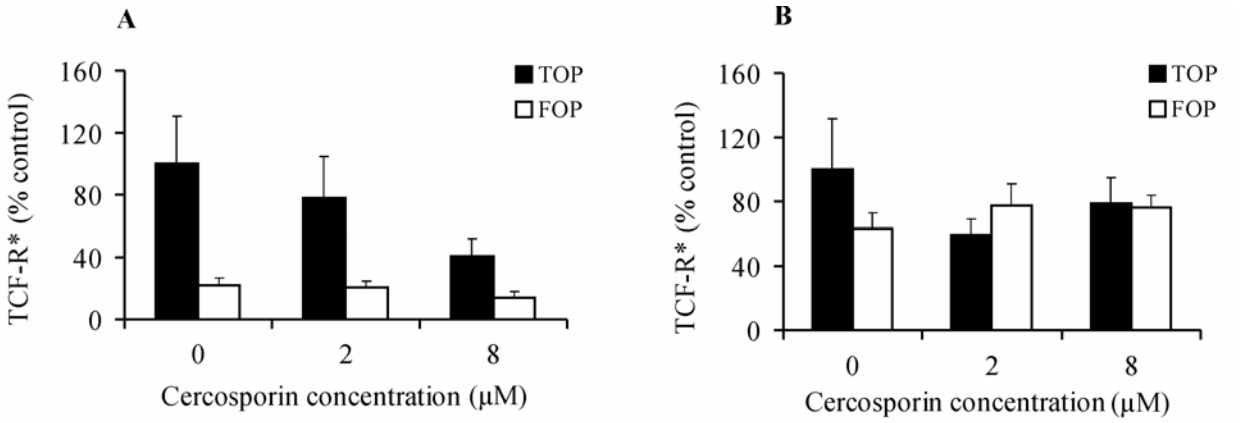

Figure 7. *TCF-reporter activity in P114* ***(A)*** *and CMT-U335* ***(B)*** *estimated 20 h after cercosporin treatment.*

Discussion

The oncogenic potential of progestins for the mammary gland is becoming increasingly evident based on *in vivo* studies in human, mice and dogs but the mechanism of the progesterone-induced tumorigenesis is still not clearly understood. Previously, we have shown that female dogs when exposed to progestins over long period, develop mammary hyperplasia and benign tumors concomitant with high expression of growth hormone mRNA within the mammary gland [8, 9]. In mice studies, the involvement of Wnt signaling in the downstream oncogenic pathway of progestins was shown [10]. Also in dogs stabilization of β-catenin resulting in enhanced

cytoplasmic and nuclear staining has been shown using immunohistochemistry [47-49]. Taken together, these studies indicated possible involvement of multiple downstream signaling pathways either independently or synergistically delivering the mitogenic effect of progestins.

As no reports exist on the effect of progestin-treatment on Wnt signaling and expression of Wnt ligands in the dog the current study was undertaken. We therefore, analyzed the expression of Wnt ligands that are reported to be expressed in mouse or human mammary gland and their target genes in progestin-induced CMH, normal mammary tissue and spontaneous canine mammary carcinomas. Progesterone-induced mammary hyperplasia in dogs was accompanied by extremely high levels of Wnt4 expression as also found in mice exposed to progestins [7, 10]. Moreover a significant albeit to a much lower extent increase in Wnt3 and Wnt5a concentrations was found. In mice, Wnt5a was found not to be responsive to ovarian hormones *in mouse mammary gland* [50]. Interestingly in bone, GH treatment was shown to upregulate Wnt5a expression [51]. It is interesting to speculate that, observed Wnt5a expression could be though progestin-induced local production of growth hormone in the mammary gland.

Wnt3, which was also significantly stimulated in CMH is known to be highly oncogenic to mammary gland as it was identified as an insertion site for mouse mammary tumor virus [52]. It is not clear whether increased Wnt3 mRNA levels in CMH results from direct or indirect effects of progestins on Wnt3 transcription. Lower expression of Wnt2 mRNA in CMH is in concordance with reported acute repression of Wnt2 expression with the appearance of lobuloalveolar structures and termination of ducal development during the progestin dominated period in mammary gland [53]. In association with the increased Wnt expression, significant increases were also found in the expression of important target genes of the canonical Wnt signaling, CyclinD1, c-myc, Survivin and Axin2. Also increased immunoreactivity of stabilized β-catenin confirmed the induction of Wnt signaling in progesterone induced CMH. Based on this, we hypothesize that the ligand induced activation of the canonical Wnt signaling plays also in the dog an important role downstream of progestins, during mammary gland development as well as in tumor initiation.

In both human and mouse, overexpression of Wnt ligands are found to be associated with aberrant canonical Wnt pathway activation in mammary cancer (reviewed in [54]) . In the dog no

information is yet available on Wnt ligand expression in spontaneous neoplasms. Aberrant expression of Wnt ligands was found and coincided with significant overexpression of canonical Wnt signaling target genes in CMCs and enhanced cytoplasmic and nuclear immunoreactivity of stabilized β-catenin as also reported by others. CMCs overexpressed Wnt3, Wnt4, Wnt5a and Wnt5b similar to that observed human breast carcinomas [33, 34, 55]. Interestingly, Wnt7a expression was 8-fold higher in CMCs compared to normal tissue. Although Wnt7a is not normally expressed in human or mouse mammary glands, it was shown to have transforming potential in cultured mouse mammary cells [56] which makes it an interesting candidate gene for further investigation.

Wnt signaling was next studied *in vitro* using three different canine mammary cell lines. In previous microarray experiments differential expression of members of the Wnt signaling was found among these cell lines [57]. In concordance with these data, cell line P114 showed constitutive activation of the canonical Wnt pathway using a TCF reporter assay giving a TOP/FOP ratio of over 4–fold. The TOP/FOP ratio of P114 further increased after stimulation with LiCl which also resulted in increased expression of CyclinD1 and Axin2 mRNA. Cell lines CMT-U229 and CMT-U335 showed low or absent basal activity of the canonical Wnt pathway and only moderated increases after LiCl stimulation. Survivin, which is also a known target of canonical signaling was slightly inhibited in CMTs upon LiCl treatment. This could be due to a moderate cytotoxic effect of LiCl (data not shown).

Finally, the therapeutic potential of a putative inhibitor of the canonical Wnt signaling cercosporin was tested. This compound, also known as CGP049090, has been reported to inhibit Wnt signaling [39, 40]. Cercosporin treatment inhibited cell viability, Wnt target gene expression and TCF-reporter (TOP) activity. However, cercosporin also marginally inhibited FOP-reporter activity which could be due to inhibition general transcription or its non-specific effect. Inhibition of TOP-reporter activity and target gene transcription by cercosporin in colon cancer cells has been reported earlier [40], although in that study no attention was given to protect against singlet oxygen formation or effects on FOP-reporter activity. The inhibition of Wnt target gene expression despite an absent or only moderate decrease in TOP/FOP ratio may point to non-specific effect of cercosporin or TCF-independent inhibition of β-catenin signaling [58].

In conclusion the important role of progestins in canine mammary tumorigenesis is mediated directly or indirectly by the regulation of Wnt ligand expression. In spontaneous carcinomas the role of progestins may decrease in time due to loss of PR expression. Still, overexpression of partly different Wnt ligands could stimulate Wnt target genes in these tumors. The cell line P114 with a constitutive activated Wnt pathway may be used to identify specific inhibitors of Wnt signaling. The previously proposed Wnt signaling inhibitor cercosporin may be an effective inhibitor. However, it may need further chemical modification to eliminate its light sensitivity and non-specific effects on cells.

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Chapter 7

Gene expression profiles of progestin-induced canine mammary hyperplasia and spontaneous mammary tumors

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(Manuscript in preparation)

Abstract

Spontaneous mammary tumors are the most prevalent type of neoplasms in women as well as in female dogs. Although ovarian hormones estrogen and progesterone are known to play a key role in mammary tumorigenesis, conflicting reports have been obtained from *in vivo* and *in vitro* studies concerning the role of especially progesterone in mammary tumorigenesis. Prolonged exposure to high concentrations of progesterone during the unusually long luteal phase of the oestrous cycle is suspected to be the key event in canine mammary tumorigenesis. Accordingly, previous studies have shown the development of mammary hyperplasia in dogs upon prolonged progestin administration. In this study, a dog-specific cDNA microarray was used to identify oncogenic determinants in progestin-induced canine hyperplasia (CMH) and spontaneous mammary tumors (CMC) by comparing expression profiles to those obtained from mammary glands of healthy dogs. The CMH profile showed elevated expression of genes involved in cell proliferation such as PCNA, NPY, RAN and also alterations in expression of transcription factors and cell adhesion molecules. Whereas in CMC, major alterations to the expression of genes involved in cell motility, cytoskeletal organization and extra cellular matrix production was evident besides differential expression of cell proliferation inducing genes. The overall gene expression profile of CMH was related to cell proliferation where as that of CMC was associated with both cell proliferation as well as neoplastic transformation. In conclusion, our findings support a strong cell proliferation inducing potential of progestins in the canine mammary gland. Moreover, deregulated genes identified in CMC are potentially involved in their malignant phenotype and may serve as potential therapeutic targets.

Introduction

Breast cancer is the most common malignancy among women in the western world [1]. The incidence of spontaneous mammary tumor is even more frequent in non-spayed female dogs compared to humans [2, 3]. Ovarian hormones, mainly estrogens and progesterone are known to play a significant role in development of mammary tumors [4, 5]. Although historically estrogen is regarded as the principal ovarian hormonal risk factor for breast cancer development, recent evidences strongly support a significant role of progestins in mammary carcinogenesis [6-8]. Unlike other mammals, dogs have only one or two estrous cycles in a year followed by a prolonged (63 days) luteal phase during which the mammary gland is continuously exposed to high concentrations of progestins [9]. Ovariectomized female dogs when exposed to progestins for 6 – 12 months developed mammary gland hyperplasia and/or benign tumors but not malignant mammary tumors [6, 8]. However, occurrence of malignant mammary carcinomas have been reported in dogs upon administration of high doses of progestins [10]. The downstream mechanism of progesterone-induced hyperplasia is not very well understood. Few studies in dogs and mouse models have implicated growth hormone (GH) and Wnt4, a ligand of the Wnt signaling pathway, as important genes in mediating the progesterone effect in target tissue [8, 11].

Progesterone receptors (PR) are expressed abundantly in the normal mammary gland but PR concentrations decrease as the tissue becomes more dysplastic/neoplastic [12]. Nearly 50% of tumors that occur in bitches are considered malignant and the majority of them are carcinomas [2]. Previous studies have indicated that 65% of canine mammary cancers as PR+; however PR status was not a predictor of clinical outcome [13]. Tumor size, histological stage, degree of differentiation and lymph node involvement are still considered as important prognostic factors for dogs as well as for human breast cancer patients [14, 15]. However, these prognostic factors may not always predict if a tumor will metastasize or not. It is very important to understand the factors/biologic processes by which tumors develop and by which processes locally confined tumors progresses to an invasive cancer. Better understanding these processes may facilitate the identification of new prognostic factors as well as therapeutic targets.

The advent of microarrays has greatly contributed to the understanding of molecular mechanisms involved in the development and progression of human breast tumors. Previous studies have successfully developed global gene expression profiles of human breast cancer and cell lines, identified molecular subtypes, predicted outcome and response to therapeutics [16-19]. There are no reports of similar studies using canine mammary tumors. Recently we developed a dog-specific cDNA microarray and used it to characterize three canine mammary cell lines originating from histologically distinct primary tumor subtypes. This study successfully identified deregulated pathways pertaining to cell line phenotype which were also found to be conserved when compared to published human breast cancer data sets [20].

In this study we investigated the altered gene expressions in progestin-induced canine mammary hyperplasia (CMH) and in spontaneous canine mammary tumors (CMC) using the canine cDNA microarray. Gene expression profiles of CMH and CMC were compared to canine mammary tissue from healthy control dogs. This study identified altered expression of genes involved in important biological processes involved in tumor development and progression. These gene expression profiles of CMH and CMC may enhance our understanding of the mechanism of progestin action in the mammary gland and of the biological processes involved in malignant carcinomas, respectively.

Materials and Methods

Canine mammary tissue

Mammary tissue from progesterone treated dogs and from healthy control dogs were obtained from frozen tissue archives and originated from our previous studies. Detailed animal experiment protocols are reported before [6, 8]. Clinical canine mammary tumors samples (CMC) were snaps frozen using liquid nitrogen and archived (at -80^0C) or fixed for paraffin embedding. Tissues originating from 8 healthy dogs, CMH originating from 8 progestin treated dogs and CMCs originating from 21 patients (Table I) were used in this study.

RNA isolation and cRNA synthesis

Total RNA from canine mammary tissues was isolated and treated with DNase using the RNeasy mini kit (Qiagen, The Netherlands) according to the manufacturer's protocol. Amplification of

Table 1. Canine mammary tumors used and their histological diagnosis.

Tissue ID	Histology	Tissue ID	Histology
BT1	Benign mixed tumor (large sized)	T16	Proliferation of connective tissue
BT2	Complex adenoma	T17	Normal like carcinoma
BT3	Lipoma, benign tumor of fat mass	T18	Ductal adenocarcinoma complex
BT4	Benign tumor ; 50% epithelium	T18d	Ductal adenocarcinoma complex
T10	Ductal adenocarcinoma simple	T3	Carcinoma
T11	Adenocarcinoma simple	T4	Carcinoma
T12	Solid Carcinoma	T5	Carcinoma
T13	Anaplastic carcinoma	T6	Multifocal mammary adenoma
T14	Carcinoma	T7	Ductal adenocarcinoma complex
T15	Carcinoma	T8	Multifocal mammary adenoma
		T9	Adenocarcinoma simple

2 µg total RNA from each sample was carried by in vitro transcription using T7 RNA polymerase as described earlier [20]. During amplification 5-(3-aminoallyl)-UTP (Ambion, The Netherlands) was incorporated into the single stranded cRNA. The quality of the total RNA and the amplified cRNA was analyzed using a bioanalyzer (Agilent technologies, The Netherlands). Three µg cRNA that met the quality criteria stipulated on Agilent bioanalyzer manual was coupled to Cy3 and Cy5 fluorophores (GE Healthcare, Belgium). A cRNA pool was prepared by adding equal quantities of amplified cRNA originating from each sample. Half of the cRNA samples from each tissue group were labeled with Cy5 and the other half with Cy3. Similarly equal quantities of pooled cRNA were also labeled with Cy5 and Cy3. Labeled cRNA was purified using ChromaSpin-30 columns (Clontech-Takara Bio, France), and dye incorporation efficiency was measured using a ND-1000 spectrophotometer (Isogen, The Netherlands). Equal quantities of labeled cRNA samples with specific activity of 2-5% dye-labeled nucleosides were used for microarray hybridization.

Microarray hybridization

Detailed protocols for the development of the canine cDNA microarray [GEO: GPL5117] are described in detail earlier. Hybridization of libeled cRNA was carried out according to a previously published protocol [20]. In brief, 2 µg of libeled cRNA was hybridized against the cRNA pooled libeled with different dye for 16-20 hours at 42^0C. Hybridized slides were washed manually and scanned using an Agilent G2565AA DNA microarray scanner (100% laser power, 30% photomultiplier tube).

Data analysis

Image analysis was carried out using Imagene 5.0 software (Biodiscovery Inc, The Netherlands). Defective spots were flagged and data was normalized based on Lowess print-tip normalization [21] by using the gene spots. Normalized data were subjected to logarithmic (base 2) transformation before further statistical analysis. Differentially expressed genes between cell lines were extracted by performing a two class unpaired t-test using significance analysis of microarray (SAM) software with the mean false discovery rate set at 5% [22]. Gene expression data were clustered and visualized using Genespring version 7 (Agilent technologies, The Netherlands). SAM generated and annotated differentially expressed genes were subjected to pathway analysis in the framework of gene ontology (GO) and signaling networks using the Panther pathway analysis tool [23]. Major pathways overrepresented by the statistically significant number of genes (random overlapping p value <0.001) were identified as important signaling pathways. Differentially expressed genes in CMH and CMC were categorized under their respective biological processes based on available functional data in the GO database. The microarray data files have been deposited in the public database.

Results

Gene expression profiles of progestin-induced hyperplasia (CMH) and spontaneous mammary neoplasias (CMC) were compared to normal mammary tissue in order to characterize the molecular basis of their phenotype. Gene expression profiles of CMH and CMC were also compared to each other. A detailed list of all differentially expressed genes identified in three

comparisons (Normal vs. CMH; Normal vs. CMC and CMH vs. CMC) with changes in their expression (fold change) is available (Supplementary Table 1). Common genes (**C**) in CMC and CMH gene expression profiles and unique genes (**U**) which are differentially expressed in CMH or CMC against the other two tissue types were identified by performing a Venn diagram analysis of gene lists from three tissue comparisons (Fig 1). All differentially expressed genes in CMH (Table 2) and CMC (Table 3) were sub grouped into gene ontology (GO) categories

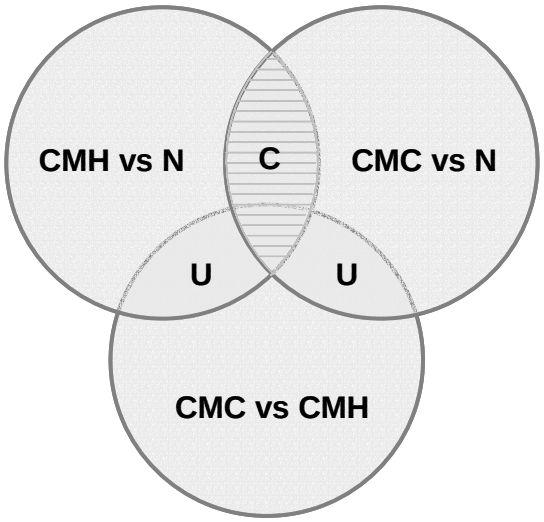

Figure 1. *Venn diagram analysis used to identify the commonly deregulated genes in CMH and CMC compared to normal tissue (C) and unique genes (U) differentially expressed in either CMC or CMH compared to the other two tissue types.*

Gene expression profile of progestin-induced canine mammary hyperplasia (CMH)

SAM analysis identified 50 upregulated and 75 down regulated (>1.8 fold) genes when the false discovery rate (FDR) was set below 5%. These genes showed changes in their expression ranging between 1.8 to 10 fold compared to normal tissue (Supplementary Table 2). The majority of these genes participated in the biological process of cell proliferation/apoptosis followed by cell adhesion/motility, transcription, amino acid biosynthesis/protein folding and ECM/cytoskeleton components and DNA integrity, respectively. Venn diagram analysis showed a cluster (C) of 13 genes that were differentially regulated in both hyperplasia and spontaneous tumors in

comparison to normal tissue and a cluster of 9 genes that were uniquely different in hyperplasia from both normal tissue and spontaneous tumors (Fig. 1). The remainder of differently expressed genes was differentially expressed in CMH compared to normal mammary tissues. Among the genes unique for progesterone-induced hyperplasia worth mentioning is a 10-fold induction of NPY expression.

Table 2. *Differentially expressed genes in progestin-induced canine mammary hyperplasia (CMH) compared to normal canine mammary tissues. (+) upregulated genes ;(-) down regulated genes; (C) commonly upregulated genes in CMH and CMC in comparison to normal tissue; (U) uniquely upregulated genes to both normal and CMC (see also Fig. 1.)*

List	Symbol	Description	Expression
Cell proliferation / cell cycle regulation / apoptosis			
C	KLF4	Kruppel-like factor 4	-
C	NME1	Non-metastatic cells 1, protein (NM23A)	+
U	NPY	Neuropeptide Y	+
	CCT2	Chaperonin containing TCP1, subunit 2 (beta)	+
	HSPB8	heat shock 22kDa protein 8	-
	NPM1	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	+
	PCNA	Proliferating cell nuclear antigen	+
	PDZK1	PDZ domain containing 1	+
	RAN	Member RAS oncogene family	+
	SSR1	Signal sequence receptor, alpha 1	+
	STMN1	Stathmin 1/oncoprotein 18	+
	TAXIBP1	T-cell leukemia virus type I binding protein 1	+
	TKT	Transketolase (Wernicke-Korsakoff syndrome)	+
DNA integrity / -repair / -recombination			
C	KPNA1	Karyopherin alpha 1 (importin alpha 5)	+
	PCNA	Proliferating cell nuclear antigen	+
	AKAP8	A kinase (PRKA) anchor protein 8	-
	FANCA	Fanconi anemia, complementation group A	-
	CETN2	Centrin, EF-hand protein, 2	-
Cell adhesion / cell motility/ proteolysis			
C	NET1	Neuroepithelial cell transforming gene 1	-
U	DPP10	Dipeptidyl-peptidase 10	+

List	Symbol	Description	Expression
U	NPY	Neuropeptide Y	+
U	TIMP2	TIMP metallopeptidase inhibitor 2	-
U	VIM	Vimentin	-
	ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	+
	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	-
	CTSC	Cathepsin C	-
	EDG1	Endothelial differentiation, G-protein-coupled receptor, 1	-
	JAM2	Junctional adhesion molecule 2	-
	PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	-
	PHLDA1	Pleckstrin homology-like domain, family A, member 1	+
	TIMP3	TIMP metallopeptidase inhibitor 3	-
Extracellular matrix related / cytoskeletal components			
	GSN	Gelsolin (amyloidosis, Finnish type)	-
	FBN1	Fibrillin 1	-
	DMKN	Dermokine	+
	DCN	Decorin	-
	CBR3	Carbonyl reductase 3	+
	ACTA2	Actin, alpha 2	-
Transcription / translation / mRNA processing			
U	EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	-
U	NCOA7	nuclear receptor coactivator 7	+
C	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	-
	TSC22D3	TSC22 domain family, member 3	-
	SNRPB2	U2 small nuclear ribonucleoprotein B	+
	PFDN5	Prefoldin subunit 5	-
	LMO4	LIM domain only 4	+
	SFRS5	Splicing factor, arginine/serine-rich 5	+
	NCOR1	Nuclear receptor co-repressor 1	-
Amino acid biosynthesis / protein folding			
C	HSP90AA1	Heat shock protein 90kDa alpha, class A member 1	+
U	ASS1	Argininosuccinate synthetase 1	+
	ASNS	Asparagine synthetase	+
	PSAT1	Phosphoserine aminotransferase 1	+
	HSPA6	Heat shock 70kDa protein 6 (HSP70B')	+

List	Symbol	Description	Expression
	HSPD1	Heat shock 60kDa protein 1 (chaperonin)	+
	HSPB8	heat shock 22kDa protein 8	-
Hormone response and fatty acid metabolism			
C	MTMR10	Myotubularin related protein 10	-
U	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	-
	ACSL6	Acyl-CoA synthetase long-chain family member 6	+
	ACOX2	Acyl-Coenzyme A oxidase 2, branched chain	-
	ELP	Endozepine-like protein type 1	-
Immune- / stress response			
C	CCL21	Chemokine (C-C motif) ligand 21	-
C	SEPP1	Selenoprotein P precursor	-
	ADORA1	Adenosine A1 receptor	+
	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	-
	CRIP1	Cysteine-rich protein 1	-
Other biological processes			
C	ROBO3	Roundabout, axon guidance receptor, homolog 3	-
C	ECSM2	Endothelial cell-specific molecule 2	-
C	FHL1	Four and a half LIM domains 1	-
C	AHNAK	AHNAK nucleoprotein (desmoyokin)	-
	AP2B1	Adaptor-related protein complex 2, beta 1 subunit	+
	CALM3	Calmodulin 3	+
	RAMP2	Receptor (G protein-coupled) activity modifying protein 2	-
	ZUBR1	Zinc finger, UBR1 type 1	-
	USP9X	USP24	+
	THBD	Thrombomodulin	-
	FBLN2	Fibulin 2	-
	TRIM2	Tripartite motif-containing 2	+
	RELN	Reelin	-
	GK	Glycerol kinase	+
	ENOPH1	Enolase-phosphatase E1	+
	CA2	Carbonic anhydrase II	+
	NNT	Nicotinamide nucleotide transhydrogenase	+
	HNRPD	Heterogeneous nuclear ribonucleoprotein D	+
	RNASEH2B	Ribonuclease H2, subunit B	+
	GNL2	Guanine nucleotide binding protein-like 2 (nucleolar)	+
	PDLIM3	PDZ and LIM domain 3	-

Gene expression profiles of canine mammary tumors (CMC)

Gene expression profiles of 21 CMC (Table 1) were compared to normal and also progestin-induced canine mammary tissues independently. SAM analysis identified 69 upregulated and 110 down regulated (>1.8 fold; FDR<5%) genes in CMC (Supplementary Table 3) compared to normal tissue. In the CMC gene expression profile, the majority of genes participated in

Table 3. *Differentially expressed genes in progestin induced canine mammary tumors (CMH) compared to the normal canine mammary tissues. (+) upregulated genes ;(-) down regulated genes; (C) commonly upregulated genes in CMH and CMC in comparison to normal tissue; (U) uniquely upregulated genes to both normal and CMH (see also Fig. 1.)*

List	Symbol	Description	Expression
Cell proliferation / cell cycle regulation			
C	KLF4	Kruppel-like factor 4	-
C	NME1	Non-metastatic cells 1, protein (NM23A)	+
U	CDC20B	Cell division cycle 20 homolog B (S. cerevisiae)	+
U	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	+
U	HTRA1	HtrA serine peptidase 1	+
U	RTN4	Reticulon 4	-
U	TSPAN3	Tetraspanin 3	+
	KLF9	Kruppel-like factor 9	-
	NFKBIA	NF-kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	-
	PDLIM4	PDZ and LIM domain 4	-
DNA integrity / -repair related			
C	KPNA1	Karyopherin alpha 1 (importin alpha 5)	+
C	KPNA1	Karyopherin alpha 1 (importin alpha 5)	+
U	CHD1L	Chromodomain helicase DNA binding protein 1-like	+
	PRKDC	DNA-dependent protein kinase catalytic	-
Cell adhesion / cell motility / proteolysis			
C	NET1	Neuroepithelial cell transforming gene 1	-
U	CCL4L1	Chemokine (C-C motif) ligand 4-like 1	+
U	CTHRC1	Collagen triple helix repeat containing 1	+
U	EDIL3	EGF-like repeats and discoidin I-like domains 3	+
U	FN1	Fibronectin 1	+

List	Symbol	Description	Expression
U	HTRA1	HtrA serine peptidase 1	+
U	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	+
U	TPM1	Tropomyosin 1 (alpha)	-
	ACTN1	Actinin, alpha 1	-
	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	-
	ADAMTS10	ADAM metallopeptidase with thrombospondin type 1 motif,10	+
	LOXL2	Lysyl oxidase-like 2	+
	PRSS23	Protease, serine, 23	+
	PSMA2	Proteasome (prosome, macropain) subunit, alpha type, 2	+
Extracellular matrix related / cytoskeletal components			
U	ACTN1	Actinin, alpha 1	-
U	COL4A1	Collagen, type IV, alpha 1	+
U	COL4A5	Collagen, type IV, alpha 5 (Alport syndrome)	+
U	COL5A2	Collagen, type V, alpha 2	+
U	MYH7	Myosin, heavy chain 7, cardiac muscle, beta	-
U	TMSB10	Thymosin, beta 10	+
	CA2	Carbonic anhydrase II	-
	CMYA3	Cardiomyopathy associated 3	-
	DAG1	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	-
	DES	Desmin	-
	LDB3	LIM domain binding protein 3	-
	LMOD2	Leiomodin 2 (cardiac)	-
	MYBPC1	Myosin binding protein C, slow type	-
	MYH8	Myosin, heavy chain 8, skeletal muscle, perinatal	-
	MYOZ1	Myozenin 1	-
	NEB	Nebulin	-
	TCAP	Titin-cap (telethonin)	-
	TMSB4Y	Thymosin, beta 4, Y-linked	+
Transcription / translation / mRNA processing			
C	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	-
U	SUB1	SUB1 homolog (S. cerevisiae)	+
U	PUM1	Pumilio homolog 1 (Drosophila)	+
U	RAB11B	RAB11B, member RAS oncogene family	+
	FHL2	Four and a half LIM domains 2	+
	GARS	Glycyl-tRNA synthetase	+
	RBM17	RNA binding motif protein 17	-
	APOBEC3	Apolipoprotein B, catalytic polypeptide-like 3F	-

List	Symbol	Description	Expression
	F		
	TAF6L	TAF6-like RNA polymerase II, p300/CBP-associated factor , 65kDa	-
Amino acid biosynthesis / protein modifications			
C	HSP90AA 1	Heat shock protein 90kDa alpha, class A member 1	+
U	PGAM2	Phosphoglycerate mutase 2 (muscle)	+
U	KDELR1	KDEL-endoplasmic reticulum protein retention receptor 1	+
	PDIA6	Protein disulfide isomerase family A, member 6	+
	KIAA0999	KIAA0999 protein	-
	DUSP28	Dual specificity phosphatase 28	-
Hormone response / fatty acid metabolism			
C	MTMR10	Myotubularin related protein 10	-
U	MGP	Matrix Gla protein	+
U	FABP2	Fatty acid binding protein 2, intestinal	-
	PANK3	Pantothenate kinase 3	+
	CDS1	CDP-diacylglycerol synthase 1	+
	APOC1	Apolipoprotein C-I	+
Immune- / stress response			
C	CCL21	Chemokine (C-C motif) ligand 21	-
C	SEPP1	Selenoprotein P precursor	-
U	FNDC3B	Fibronectin type III domain containing 3B	+
	TF	Transferrin	+
	CCDC6	Coiled-coil domain containing 6	-
	CIRBP	Cold inducible RNA binding protein	-
Other biological processes			
C	AHNAK	AHNAK nucleoprotein (desmoyokin)	-
C	ECSM2	Endothelial cell-specific molecule 2	-
C	FHL1	Four and a half LIM domains 1	-
C	ROBO3	Roundabout, axon guidance receptor, homolog 3	-
U	LDHB	Lactate dehydrogenase B	+
U	TMEM16A	Transmembrane protein 16A	-
U	TNNT2	Troponin T type 2 (cardiac)	-
U	VIPR2	Vasoactive intestinal peptide receptor 2	-
	TXNIP	Thioredoxin interacting protein	-
	ANKRD37	Ankyrin repeat domain 37	+
	ARF1	ADP-ribosylation factor 1	-

List	Symbol	Description	Expression
	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	-
	ATP5G1	ATP synthase, H+ transporting, subunit C1	+
	CAB39L	Calcium binding protein 39-like	-
	CKMT1A	Mreatine kinase, mitochondrial 1A	-
	CMYA5	Cardiomyopathy associated 5	-
	COX7A2	Cytochrome c oxidase subunit VIIa polypeptide 2	+
	CSPG5	Chondroitin sulfate proteoglycan 5 (neuroglycan C)	+
	GOPC	Golgi associated PDZ and coiled-coil motif containing	-
	HNRPD	Heterogeneous nuclear ribonucleoprotein D	-
	MB	Myoglobin	-
	MYL2	Myosin, light chain 2, regulatory, cardiac, slow	-
	MYOM1	Myomesin 1 (skelemin) 185kDa	-
	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	-
	SLN	Sarcolipin	-
	TTN	Titin	-
	SLC4A1	Solute carrier family 4, anion exchanger, member 1	

biological processes regulating ECM/cytoskeletal component biogenesis and remodeling. Among them, genes involved in cell adhesion, motility, proliferation and extra cellular matrix components such as collagen were also unique in the CMC gene expression profile (Table 3). When compared to the CMH gene expression profile, the CMC profile showed higher representations of genes which are involved in ECM/cytoskeleton organization whereas the CMH profile contained a higher number of genes involved in cell proliferation/apoptosis (Fig 2).

Pathway analysis

All differentially expressed genes in CMH and CMC against normal mammary glands were separately compared to a (human) reference list available in the Panther pathway tool in order to identify over representation of genes under various biological pathways. The majority of genes in each gene list were, however, not categorized under any pathway and CMH showed no statistically significant over representation of genes under any pathway. In the CMC gene list, however, integrin signaling, inflammation mediated by chemokine and cytokine, cytoskeletal/Rho GTPase and Wnt signaling pathways were over represented by a statistically significant number of genes (random overlapping $p < 0.01$) in the same order.

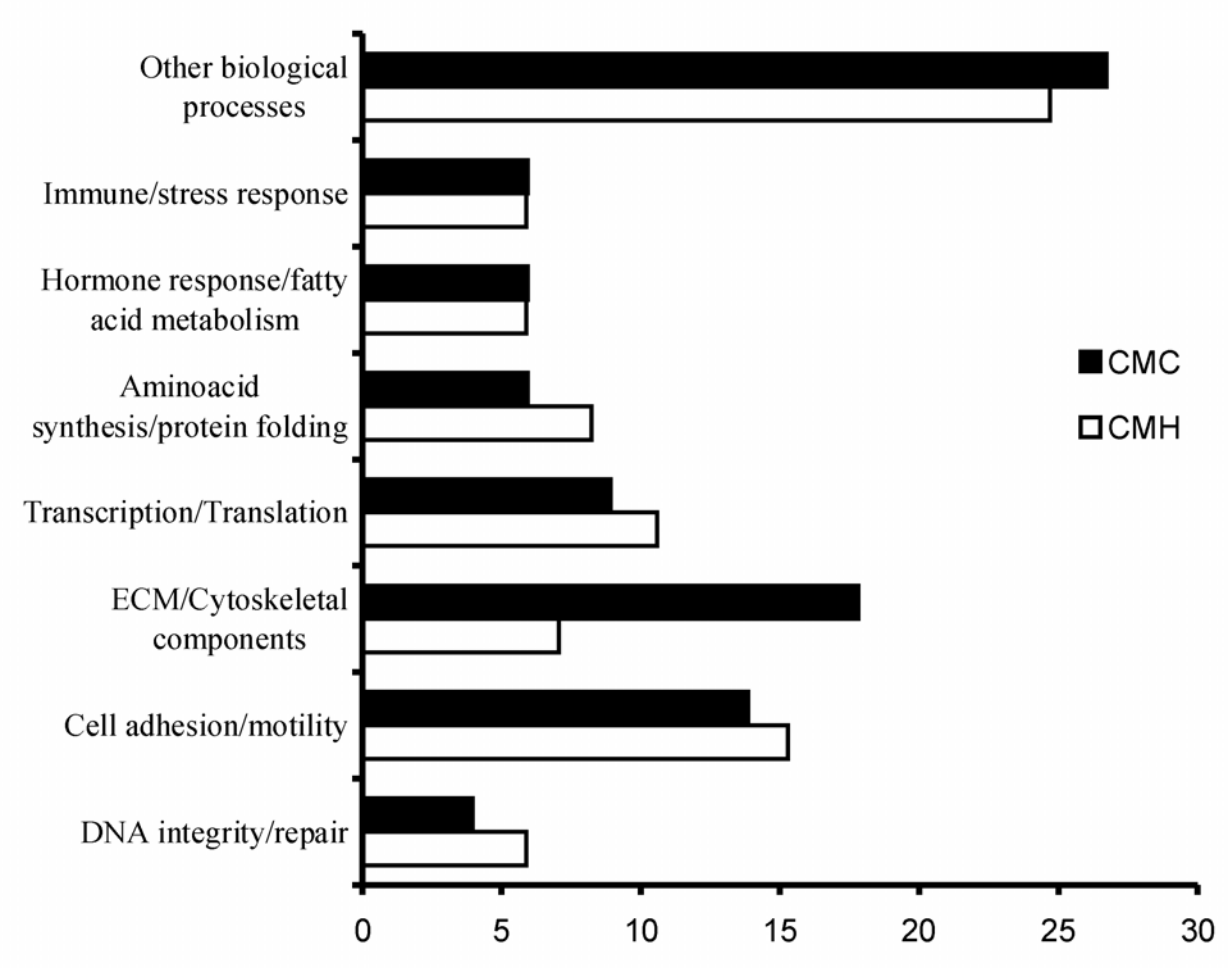

Figure 2. *Functional classification of genes differentially expressed in progestin-induced canine mammary hyperplasia (CMH) and in spontaneous canine mammary tumors (CMC) in comparison to the normal mammary gland. On the x-axis number of genes is expressed as a ratio (%) to the total number of differentially expressed genes within the same functional annotation group.*

Discussion

Reports based on in vitro and in vivo studies on proliferative effect of progestins on human mammary epithelium are conflicting. In vivo the proliferative activity of mammary epithelium is highest during the luteal phase of the estrous/menstrual cycle which co-insides with high serum estrogen and progesterone concentrations. Hormone replacement therapy (HRT) containing estrogens in combination with progestins in postmenopausal women increased the risk of breast cancer development when compared to estrogens only [7]. Similarly, ovariectomized dogs when exposed to progestins developed mammary hyperplasia and benign tumors [6, 8]. To further elucidate the role of progestins in mammary tumor development, we compared the gene expression profiles of progestin-induced CMH and spontaneous CMC to that of normal canine mammary tissue.

The CMH gene expression profile was dominated by altered expression of genes involved in cell proliferation and cell adhesion/cell motility which are key biological processes required for the cell homeostasis and if deregulated may lead to cancer. Interestingly, among 13 differentially expressed cell proliferation related genes, all upregulated genes were either stimulators of cell proliferation or inhibitors of apoptosis and the down regulated genes were either growth inhibitors or positive regulators (based on GO description). PCNA, a well known cell proliferation marker was upregulated in CMH, similar to the overexpression of PCNA in mammary epithelium of women who received HRT (E or P+ E) [24]. NPY, which was uniquely (10 fold) upregulated in CMH, is known to be expressed in the normal mammary gland and to be overexpressed in many human tumors (reviewed in [25]). Physiologically, among many other functions, NPY is known to regulate the LH and LHRH surge from the hypothalamus [26, 27]. Suppression of NPY in ovariectomized monkeys impaired the progesterone-induced LHRH release [28]. Also an earlier study using murine endometrial epithelial cells showed that NPY is strongly induced in response to sex steroids [29]. Taken together these studies and the overexpression of NPY in CMH indicate a key role for NPY in mediating the progesterone effect in the mammary gland.

RAN is another interesting gene overexpressed in CMH which is also reported to be involved in cell proliferation and cancer progression [30]. RAN is known to be involved in nucleocytoplasmic shuttling of steroid receptors [31] and may be another key gene involved in

progesterone signaling. Among the down regulated genes, KLF4, an epithelial transcription factor that is known to inhibit the β-catenin signaling and is often downregulated in colorectal cancers [32]. As Wnt4, ligand and activator of Wnt signaling is also strongly induced in the canine mammary gland upon progestin treatment (manuscript in preparation), observed KLF4 down regulation in CMH may indicate an enhanced Wnt signaling in progesterone-induced mammary hyperplasia. In this context, it is relevant to mention that, Wnt4 is also induced in mouse mammary gland upon progestin treatment [11].

Alterations in expressions of genes involved in cell adhesion, ECM and cytoskeletal organization are important for ductal development/side-branching in the mammary gland and also in oncogenic transformation. Amplified expression of proteolysis or cell motility promoting proteins like DPP10, NPY, and ADAMTS7 and down regulation of the metalloproteinase inhibitors (TIMP1, TIMP2) may facilitate side-branching and proliferation. However, the transforming effect due to overexpression of these genes in CMH may have been hampered by the down regulation of known transforming/proteolytic genes such as NET1, VIM and PHLDA1 [33, 34]. A similar pattern of expression of genes that are involved in cellular transcription and translation machinery was observed in CMH. FOS, an oncogenic transcription factor which is amplified in various tumors was found downregulated in CMH [35]. Lower expression of the estrogen receptor target gene NCOR1 and overexpression of the nuclear co-activator NCOA7 [36, 37] may also potentially modify the activated PR effects.

Next, we measured gene expression profiles of spontaneous CMC to identify altered expression of genes involved in key biological pathways as described in the case of CMH. Interestingly, in CMC a large number of genes encoding ECM proteins (collagens) were upregulated and proteins involved in cytoskeletal organization biogenesis were down regulated (DES, ACTN1, NEB, and DAG1). Experimental evidence in various caner models have indicated a loss of expression of cytoskeletal components like ACTN1 and DAG1 can lead to malignant transformation [38, 39]. Collagens, which are ligands of integrin signaling are believed to be passive components of malignant transformation, however, the significance of their overexpression in tumors is yet to be elucidated.

Many genes involved in cell motility and proteolysis were overexpressed in CMC. Among them, genes known to be involved in tumor invasion such as FN1, a target of WNT signaling [40], SPARC and CTHRC1 [41, 42] were uniquely upregulated in canine mammary tumors. CMC also overexpressed the oncogenic gene FHL2 [43] among many overexpressed genes involved in cell transcription and translation machinery. The overall gene expression pattern in CMC indicated a significant molecular alteration towards malignant transformation. Similar to CMH, many genes that are involved in cell growth control were deregulated in CMC. Among them, CTNNB1 (β-catenin), a key mediator of canonical Wnt signaling [44] was upregulated and two inhibitors of β-catenin action ANKRD15 [45] and KLF4 were down regulated. Together with the overexpression of FN1, this indicates an increased activity of the canonical Wnt signaling in CMC. Other negative regulators of cell proliferation (NFKBIA and PDLIM4) were also down regulated in CMC giving them an overall growth advantage.

Pathway analysis of CMH profile did not identify any overrepresentation of genes in important cellular signaling pathways. However, the majority of genes were not categorized under any pathway due to lack of information regarding their signaling pathways. In contrast to CMH, pathway analysis of the gene expression profile of CMC identified significant overrepresentation of genes that are involved in the integrin signaling followed by genes involved in immune response-related signaling and Wnt signaling. These results are comparable to our earlier observation in canine mammary cell lines, and in human breast cancer and cell line pathway profiles [20].

In summary, CMH gene expression profiles indicate a strong cell proliferation inducing effect of progestin. Alterations in the expression pattern of transcription factors, genes involved in maintaining DNA integrity and cell motility may also indicate early stages of malignant transformation. It is worthwhile to mention in this context that benign hyperplasia is an important risk factor for breast cancer in women [46] The CMC gene expression profile indicated a more pronounced expression of genes involved in malignant transformation (cytoskeletal components/ ECM/ cell motility), in addition to amplified expression of many proliferation stimulating genes. The gene expression data of CMH further reiterates the pronounced tumorigenic effects of

progestins in canine mammary gland upon prolonged exposure and it may have implications for human breast cancer research.

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Chapter 8

Summarizing discussion and conclusions

A better molecular characterization of mammary tumor cell lines may help to identify new tumor markers and new therapeutic targets. Basal gene expression profile of tumor cell lines can also assist in judging the cell line suitability for *in vitro* experiments. There are not many well-defined cell line models available for canine mammary cancer research. In **Chapter 3,** we developed gene expression profiles of three canine mammary tumors cell lines (CMT) originating from histological distinct primary tumors. Meanwhile, a newly developed canine cDNA microarray was successfully validated by quantifying the expression of selected differentially expressed genes using RT-qPCR. Altered gene expression in CMTs could partially explain the distinct biological behaviors of individual cell line such as growth rate, invasiveness and preserved characteristics of their primary tumors of origin. Based on the level of representation of differentially expressed genes the Wnt, cell cycle, and integrin signaling pathways were identified as putative altered pathways in cell lines P114, CMT-U229 and CMT-U335, respectively. The biological significance of the gene expression changes observed in the cell lines becomes more meaningful upon further experimental validation. In **Chapter 3,** high expression of response gene to complement 32 (RGC32) and factor B (BF) in cell line CMT-U229 was discussed in relation to its high growth rate. Based on this idea, in **Chapter 4,** the role response gene to complement 32 (RGC32) and factor B (BF) in cell proliferation rate was investigated. According to our initial hypothesis, RGC32 and BF were confirmed to be the stimulators of cell growth. Further, amplified activity of cell division cycle 2 (CDC2) was found to be the cause of overexpression of RGC32 and BF in CMT-U229. The level of expression of BF and RGC32 mRNA showed good correlation in spontaneous canine mammary tumors and cell lines suggesting a possible CDC2 mediated co-regulation of their expression. Amplified CDC2 activity/expression is frequently found in various human cancer tissues [1-3]. Elevated expression of both RGC32 and BF transcripts in tumors could serve as a marker for amplified CDC2 activity in those tumors. However, an experiment correlating the CDC2 activity to the level of RGC32 and BF expression in a larger panel of tumors from a variety of tissues may further strengthen this hypothesis. Further, questions regarding the exact mechanism of regulation of RGC32 and BF expression by CDC2 kinase are yet to be addressed. A small molecule inhibitor of CDC2, (CGP-74514A-HCl) used in this study specifically inhibited CDC2 activity as well as the expression level of CDC2,

RGC32 and BF transcripts at a nanomolar concentration. This compound has a potential therapeutic value for patients with mammary tumors characterized by amplified CDC2 activity.

Similarly, gene expression profiles of CMTs also suggested a possible alteration in canonical Wnt pathway in P114 cell line. In **Chapter 6,** all three cell lines were tested for their basal canonical Wnt pathway activity using a TCF-reporter assay. Out of three tested CMTs, one (P114) showed constitutive activation of Wnt signaling. This cell line could be used in the future to identify specific inhibitors of the canonical Wnt signaling. Based on this idea a previously proposed canonical Wnt signaling inhibitor cercosporin (CGP049090)[4] was tested for its inhibitory potential. It was concluded that cercosporin may need further chemical modifications to eliminate its light sensitivity and non-specific effect on cells.

Wnt signaling is known for its regulatory role in early development, adult tissue maintenance, cell fate decisions, stem cell renewal and tumorigenesis of various tissues. More than 60 % of human breast carcinomas analyzed show stabilized nuclear β-catenin staining, an indicator of active canonical Wnt signaling [5, 6]. The cause of this activation is proposed to be overexpression of Wnt ligands. Mutation in the tumor suppressor APC is an established event causing the frequently observed constitutive activation of Wnt signaling in human colon carcinomas. Such mutations are very rarely observed/absent in human breast tumors. In **Chapter 6** we investigated the expression levels of key Wnt ligands and target genes in canine mammary tumors. Overexpression of these genes as well as stabilized β-catenin immunoreactivity suggested an elevated canonical Wnt pathway activity in canine mammary tumors compared to normal tissue. Increased expression of Wnt ligands and decreased expression of endogenous Wnt pathway inhibitors could be the mechanism of pathway activation (stabilized β-catenin) in canine mammary tumors. Similar ligand dependent activation canonical Wnt signaling has been reported in malignant human breast cancer cell lines [7].

In addition to the observed activation of Wnt signaling, it is also very informative to develop a global gene expression profile of spontaneous tumors. Many microarray studies have successfully characterized human breast carcinomas and cell lines [8-10]. Using a similar approach we characterized the canine mammary tumors to identify altered gene expression compared to normal tissue **(Chapter 7)**. The majority of differentially expressed genes in

mammary tumors were already known for their role in various cellular processes related to invasive potential of tumor cells such as cell motility, cytoskeletal reorganization, cell adhesion and invasion. Expression of cell proliferation inducing oncogenes was also found to be elevated in canine mammary tumors. Pathway analysis revealed overrepresentation of integrin and Wnt signaling in canine mammary tumor profile which is also comparable to what was observed in canine mammary cell line and human breast tumors profiles (**Chapter 3**). This canine mammary tumor profile can be used for identifying new markers and therapeutic targets. This data set can also be used as a classifier for large-scale microarray studies aiming to classify canine mammary tumors into molecular subtypes.

As discussed in **Chapter 2,** progesterone is proven to be a major risk factor for canine mammary tumor development. Downstream effectors or signaling of progesterone action in mammary gland is not clearly elucidated. In human breast cancer studies, the oncogenic potential of progesterone is still being debated due to contradicting reports regarding progestin action in various experimental set ups. Nevertheless, hormone replacement treatment trials in postmenopausal women indicated significantly a higher risk of developing breast tumors in case of estrogen in combination with progestin compared to estrogen alone [11]. Even though mammary tumors incidence in non-spayed female dogs is nearly three times higher compared to women, early ovariectomy is the best available preventive strategy and surgery is being the most effect treatment in dogs [12]. The overall 2-year post-surgery survival has been reported to be between 25% and 40% with a mean survival time ranging from 4 to 17 months [13]. There is a need for improved understanding downstream effectors of progesterone which may add valuable information on early stages of canine mammary tumorigenesis. This may also facilitate identification of new markers as well as new potential therapeutic (preventive) targets. Detailed information regarding all putative downstream targets of progestin in mammary gland is of importance in this context.

Progesterone is known to act in a paracrine manner by inducing local growth factors in a subset of mammary cells. Previous studies reported the progestin-induced mammary gland derived GH excess in the dogs. **Chapter 5** deals with the role of progestin-induced mammary derived GH in the canine mammary gland and endometrium. This study confirmed the previously

reported induction of GH mRNA in progestin-induced canine hyperplasia (CMH) using a more sensitive quantitative RT-PCR. A recently concluded study indicated that *in vitro* progesterone treatment alone had no direct stimulatory effect on GH transcription using a GH promoter-luciferase construct[14]. It is therefore suggested that progesterone may induce GH expression in the mammary gland indirectly through other signaling pathways.

In the mouse mammary gland, progesterone is known to induce high expression of Wnt4 in mouse mammary epithelial cells [15]. In **Chapter 6** we confirmed a similar phenomenon in the canine mammary gland. In addition to Wnt4, other ligands (Wnt3 and Wnt5a) and target genes of the canonical Wnt signaling were found to be elevated in mammary tissue of progestin-treated dogs. Further, increased β-catenin immunoreactivity in the mammary gland suggested elevated activity of canonical Wnt signaling in progestin-treated animals. Wnt proteins are known to be expressed in mouse mammary glands during pregnancy which also coincides with high concentrations of plasma progesterone [15]. Taken together, ligand-induced activation of the canonical Wnt signaling could be one of the downstream modes of action of progesterone in the mammary gland. Wnt proteins may also have a role in progestin-induced GH excess in the mammary gland. Involvement of other unknown growth factors delivering progestins action, however, cannot be excluded. In **Chapter 7,** progestin induced gene expression changes were quantified by microarray analysis. Data indicated a clear induction of many cell proliferation-inducing genes in CMH, such as proliferating cell nuclear antigen (PCNA), neuropeptide Y (NPY) and the ras-related nuclear protein (RAN). Hormone replacement therapies (HRT) in postmenopausal women involving estrogens in combination with progestins showed a higher risk of breast cancer development compared to estrogen alone. Breast tissue derived from these patients also showed high immunoreactivity for PCNA among other quantified genes [16]. These experimental evidences clearly indicate the conserved oncogenic role of progestins in the mammary gland. Many genes known to be involved in steroid hormone signaling as well as Wnt signaling were found differentially expressed in CMH. These genes are also potential candidates for the progestin-mediated action in the mammary gland. It is worthwhile to further investigate the mechanism regulating these genes in CHM using *in vitro* experimental models. Such studies

will definitely enhance our understanding on progestin-induced pathogenesis of the mammary gland.

Mammary gland undergoes sequential tissue remodeling during puberty, estrous cycle and pregnancy to generate new tissue structures. Stem cells are known to play crucial role in developmental stages of mammary gland as well as in tumorigenesis. Prospective, mouse mammary stem cells isolated through identification using cell surface markers demonstrated to reconstitute an entire mammary gland comprising all mature epithelial cell types. These cells were also capable of serial transplantation, thus possessing stem cell features [17]. As discussed earlier, steroid hormones act in a paracrine fashion in the mammary gland delegating different functions to locally produced factors. Among them, Wnt proteins are worthwhile to be mentioned in this context. Many studies have indicated the ability of Wnt proteins to promote self-renewal of stem cells and thus the maintenance of stem cell fate. According to a proposed hypothesis [18], stem cell niche undergoes changes depending on hormonal milieu during different stages of mammary tissue remodeling. Repeated hormonal stimulation of stem cells and their niches during the course of repeated estrous cycles could be an important early event in mammary tumorigenesis. Taken together, prolonged exposure to progesterone during canine luteal phase and/or pseudopregnancy, through locally produced growth factors such as Wnt, GH, and NPY may stimulate mammary stem cells. This may lead to mammary hyperplasia as an early event of oncogenic transformation. Isolation of quiescent mammary stem cells and further characterization of their interaction with progesterone, GH, Wnt and other growth factors using *in vitro* and *in vivo* experimental systems could make new inroads into early stages of mammary tumorigenesis. This may help to develop effective preventive and or therapeutic strategies to contain neoplastic transformation of a hyperplastic mammary lesion.

Finally, gene expression profiles of canine mammary cell lines, canine mammary tumors and progestin-induced hyperplasia, identified large number of differentially expressed genes with little/no functional information. Using well characterized cell lines (**Chapter 3**), gaining information regarding functional role of these genes in a similar approach as elaborated in **Chapter 4,** may greatly enhance our understanding of tumor biology.

Samenvatting en conclusies

Een betere moleculaire karakterisering van melkkliertumor cellijnen helpt bij het identificeren van nieuwe tumormerkers en nieuwe aangrijpingspunten voor therapie. De basale genexpressie profielen van tumorcellijnen kan ook behulpzaam zijn bij de beoordeling van de geschiktheid van een cellijn voor *in vitro* experimenten. Voor het onderzoek naar melkkliertumoren bij de hond zijn niet veel goed gekarakteriseerde cellijnen voor handen. In **hoofdstuk 3** wordt de ontwikkeling van gen expressieprofielen van drie honden melkkliercellijnen (CMT) met een verschillende histologische achtergrond beschreven. Met behulp van een kwantitatieve RT-PCR voor een geselecteerd aantal differentieel tot expressie komende genen werd een nieuw ontwikkelde honden cDNA array succesvol gevalideerd. De verschillen in genexpressie tussen de CMTs konden gedeeltelijk het verschil in biologisch gedrag zoals groeisnelheid, invasiviteit en oorspronkelijke histologisch karakter van de primaire tumor worden verklaard. Op basis van verschillen in expressie konden de signaaltransductiepaden van Wnt, de celcyclus en integrine als vermoedelijke veranderingen in achtereenvolgens de cellijnen P114, CMT-U229 en CMT-U335 worden geïdentificeerd. Voor het biologische belang van de veranderde genexpressie in de cellijnen was verdere experimentele bevestiging noodzakelijk. In **hoofdstuk 3** wordt de hoge expressie van het RGC32 gen (response gene to complement 32) en het gen coderend voor factor B (BF) in cellijn CMT-U229 bediscussieerd in relatie tot de hoge groeisnelheid. Op basis hiervan wordt in **hoofdstuk 4** de rol van RGC32 en BF in verhoogde celdeling onderzocht. In overeenstemming met de gestelde hypothese werd de rol van RCG32 en BF bevestigd als genen die de celgroei stimuleren. De oorzaak van de hoge expressie van RGC32 en BF werd veroorzaakt door toegenomen activiteit van het CDC2 (cell division cycle 2) enzym in cellijn CMT-U229. De mate van expressie van BF en RGC32 vertoonde een goede correlatie in spontane melkkliertumoren en cellijnen wat suggereert dat hun beider expressie door CDC2 wordt gereguleerd. Toegenomen CDC2 activiteit/expressie wordt vaak gevonden in een aantal humane tumoren[1-3]. De verhoogde expressie van zowel RGC32 als BF in tumoren kan dienen als een merker voor verhoogde CDC2 activiteit. Onderzoek naar de correlatie van CDC2 activiteit in een groter aantal tumoren van verschillende weefsels kan deze hypothese versterken. Verder moeten

nog vragen worden beantwoord rond het exacte mechanisme hoe CDC2 de expressie van RCG32 en BF reguleert. Een remmer van de CDC2 activiteit (CGP-74514A-HCl) die in onze studie werd gebruikt remde de CDC2 activiteit en de expressie van CDC2, RGC32 en BF in nanomol concentraties. Deze stof heeft potentieel therapeutische waarde bij patiënten met melkkliertumoren die worden gekarakteriseerd door verhoogde CDC2 activiteit.

Uit de genexpressie profielen komt ook een mogelijke verandering van het canonieke Wnt pad in cellijn P114 naar voren. In **hoofdstuk 6** wordt de basale activiteit van het Wnt pad beschreven in de drie gebruikte cellijnen gemeten met een TCF-reporter bepaling. Van deze drie vertoonde P114 een constitutieve activering van Wnt signalering. Deze cellijn is daardoor bruikbaar voor onderzoek naar specifieke remmers hiervan. Op grond hiervan werd de door anderen voorgestelde remmer van het canonieke Wnt pad cercosporin (CGP049090)[4] getest voor het remmend vermogen. Geconcludeerd werd dat cercosporin verdere chemische veranderingen nodig heeft ten einde de bijwerkingen van lichtgevoelige radicaal vorming en niet specifieke effecten op cellen te elimineren.

Wnt signalering is bekend in verband met de rol in de regulering van vroege ontwikkeling, onderhoud van ontwikkelde weefsels, beslissingen rond celdood, vernieuwing van stamcellen en tumorvorming in verschillende weefsels. Meer dan 60% van de onderzochte humane borstkankerweefsels vertonen gestabiliseerde kernkleuring voor β-catenine wat duidt op een actieve canonieke Wnt signalering [5, 6]. De oorzaak van deze activering wordt geacht te worden veroorzaakt door overexpressie van Wnt eiwitten. Bij kanker van het colon wordt activering van het Wnt pad veelal veroorzaakt door mutaties van de tumorsuppressor APC. Zulke mutaties zijn erg zeldzaam of zelfs afwezig in borstkanker bij de mens. In **hoofdstuk 6** wordt onderzoek beschreven naar de expressie van Wnt liganden in melkkliertumoren bij de hond. Overexpressie van deze genen samen met een gestabiliseerde immunoreactiviteit voor β-catenin wijzen op een toegenomen canonieke Wnt activiteit in melkkliertumoren ten opzichte van normaal melkklier weefsel. Toegenomen expressie van Wnt liganden en afgenomen expressie van endogene remmers van het Wnt pad kunnen de oorzaak zijn van de activering hiervan (gestabiliseerde β-catenine) in melkklier tumoren van de hond. Een soortgelijke ligand afhankelijke activering van het Wnt pad is beschreven in humane borstkanker cellijnen[7].

In aanvulling op de waargenomen activering van het Wnt pad is het ook zeer informatief om een allesomvattend genexpressie profiel van spontaan voorkomende tumoren te ontwikkelen. In veel microarray studies zijn met succes humane borstkanker weefsels en cellijnen gekarakteriseerd[8-10]. In een gelijke benadering hebben we honden melkklier tumoren gekarakteriseerd om veranderde gen-expressie in tumoren ten opzichte van gezond weefsel te identificeren **(hoofdstuk 7)**. In meerderheid zijn de verschillend tot expressie komende genen bekend voor hun rol in cellulaire processen in relatie tot het vermogen tot invasiviteit van tumorcellen zoals beweeglijkheid, reorganisatie van het cytoskelet, het aan elkaar hechten van cellen en invasiviteit. Ook werd expressie gevonden van oncogenen die de celvermeerdering stimuleren verhoogd aangetroffen in melkklier tumoren van de hond. Analyse van signaal-transductie paden vertoonde een toegenomen aanwezigheid aan van integrine en Wnt signalering in het profiel van honden melkklier tumoren, vergelijkbaar met wat is waargenomen in genexpressie profielen van honden melkklier cellijnen en humane borstkanker **(hoofdstuk 3)**. Het tumorprofiel van de hond is bruikbaar voor het identificeren van nieuwe merkers en therapeutische aangrijpingspunten. Tevens kunnen deze data gebruikt worden om met een op grotere schaal toegepaste microarray studie honden melkkliertumoren in moleculaire subtypen te rangschikken.

Zoals besproken in **hoofdstuk 2** is bewezen dat progesteron een belangrijke risicofactor is voor de ontwikkeling van borstkanker bij de hond. De aangrijpingspunten voor progesteron in de melkklier zijn niet duidelijk opgehelderd. In humane borstkanker studies is het tumorbevorderende potentieel nog steeds aan discussie onderhevig vanwege tegenstrijdige berichten met betrekking tot de werking van progestagenen in verschillende onderzoekopzetten. Niettemin onderzoeken naar hormoon substitutie behandeling van postmenopausale vrouwen geven een verhoogd risico voor de ontwikkeling van borstkanker in de combinatie van progesteron en oestrogenen in vergelijking tot alleen oestrogenen[11]. Melkklier tumoren bij de hond hebben zelfs een drie keer hogere incidentie in vergelijking met de vrouw. Een vroege ovariectomie is de beste preventie strategie terwijl chirurgie de meest effectieve behandeling is bij honden[12]. De totale overleving 2 jaar na chirurgie ligt tussen de 25 en 40% met een gemiddelde overlevingstijd van 4 tot 7 maanden[13]. Er is de behoefte aan een beter begrijpen van de manier

waarop progesteron aangrijpt waardoor waardevolle informatie wordt verkregen voor vroege stadia van tumorvorming in de melkklier van de hond. Dit kan ook het identificeren vergemakkelijken van nieuwe merkers en mogelijk nieuwe terapeutisvhe (preventieve) doelen. Gedetailleerde informatie over alle mogelijke doelen van progesteron in de melkklier is belangrijk in dit verband.

Van progesteron is bekend dat het op een paracriene manier werkt doordat het de vorming van lokale groeifactoren induceert in een subset van melkklier cellen. Vroegere studies hebben de progesteron geïnduceerde overmatige groeihormoon productie in de melkklier aangetoond. **Hoofdstuk 5** beschrijft de rol van progesteron gestimuleerde mammaire GH productie in de melkklier en het endometrium. Deze studie bevestigt de gerapporteerde inductie van GH mRNA expressie in door progesteron gestimuleerde melkklier hyperplasie (CMH) bij de hond gemeten met een gevoelige kwantitatieve RT-PCR. Recentelijk werd geconcludeerd dat *in vitro* progesteron behandeling van melkkliercellijnen niet leidt tot een stimulerend effect van een GH promoter-luciferase construct[14]. Vanuit deze studies wordt gesuggereerd dat progesteron de expressie van GH in de melkklier op een indirecte wijze stimuleert volgens andere signaal transductie wegen.

In de melkklier van de muis stimuleert GH een hoge expressie van Wnt 4 in melkklier epitheel[15]. In **hoofdstuk 6** wordt een soortgelijk fenomeen bevestigd in de melkklier van de hond. In aanvulling op Wnt4, werd gevonden dat ook andere Wnt liganden (Wnt3 en Wnt5a) en doelgenen van de canonieke Wnt verhoogd tot expressie komen in de melkklier van met progesteron behandelde honden. Verder duidde een verhoogde β-catenine kleuring in de melkklier op een toename van het canonieke Wnt pad bij progesteron behandelde dieren. Wnt eiwitten zijn bekend om hun expressie in de melkklier van de muis gedurende de zwangerschap, een periode die gepaard gaat met hoge plasma progesteron concentraties[15]. Alles tezamen is de ligand gestimuleerde activering van het canonieke Wnt pad één van de manieren waarop progesteron de melkklier beïnvloedt. Wnt eiwitten kunnen ook een rol spelen bij de progesteron gestimuleerde mammaire GH overmaat. De betrokkenheid van andere onbekende groeifactoren die het progesteron effect afgeven kunnen niet worden uitgesloten. In **hoofdstuk** 7 worden de progesteron geïnduceerde veranderingen in gen expressie onderzocht door analyse met

microarrays. Hieruit blijkt een duidelijke toename van veel genen betrokken bij cel proliferatie in progesteron geïnduceerde hyperplasie zoals PCNA, NPY en het RAS-gerelateerde kern eiwit RAN. Hormoon substitutie therapieën van postmenopausale vrouwen met een combinatie van progestagenen en oestrogenen vertonen een verhoogd risico op de ontwikkeling van borstkanker in vergelijking met alleen oestrogenen. Melkklierweefsel verkregen bij deze patiënten laten ook een verhoogde immunoreactiviteit zien voor PCNA naast andere gemeten genen[16]. Dit experimenteel verkregen bewijs wijzen duidelijk op een over de soorten bewaard gebleven rol van progesteron in de oncogenese. Veel genen die bekend zijn uit de signaal transductie door steroïden en ook Wnt komen verschillend tot expressie in melkklier hyperplasie bij de hond. Deze genen zijn goede kandidaten voor progesteron effecten in de melkklier. Het is de moeite waard om dit mechanisme verder te bestuderen gebruik makend van experimentele *in vitro* modellen teneinde ons begrip van progesteron geïnduceerde pathogenese van de melkklier te vergroten.

De melkklier ondergaat een sequentiële transformatie gedurende de puberteit, de geslachtscyclus en zwangerschap ten einde nieuwe structuren te ontwikkelen. Stamcellen spelen een cruciale rol in de ontwikkelingsfasen van de melkklier evenals bij de tumorvorming. Uit muizen stamcellen geïsoleerd uit de melkklier kan opnieuw een volledige melkklier zich ontwikkelen. Deze cellen zijn ook geschikt voor seriële transplantatie en hebben dus stamcel eigenschappen[17]. Zoals eerder beschreven werken steroid hormonen op een paracriene manier in de melkklier waardoor verschillende functies worden gedelegeerd aan lokaal geproduceerde factoren. In dat verband zijn de Wnt eiwitten het vermelden waard. Vele studies hebben aangegeven dat Wnt eiwitten het vermogen hebben om de zelf vernieuwing en instandhouding van stamcellen te bevorderen. Volgens de voorgestelde hypothese[18], ondergaat de directe stamcel omgeving veranderingen afhankelijk van de hormonale omgeving gedurende verschillende fasen van melkklier reemodellering. Herhaalde hormonale stimulatie van stamcellen en hun compartiment gedurende de loop van herhaalde geslachtcycli kan een belangrijke vroege gebeurtenis zijn in de tumorontwikkeling in de melkklier. Langere blootstelling aan progesteron gedurende de luteale fase in de geslachtscyclus van de hond en/of gedurende schijnzwangerschap met als gevolg lokale productie van groeifactoren zoals de Wnt eiwitten, groeihormoon en NPY kunnen deze melkklier stamcellen stimuleren. Dit kan dan leiden tot melkklier hyperplasie als een

vroege gebeurtenis van tumorigene transformatie. Isolatie van inactieve melkklier stamcellen en verdere karakterisering van hun interactie met progesteron, GH, Wnt en andere groeifactoren met zowel *in vitro* als *in vivo* experimentele systemen kunnen nieuwe wegen verhelderen van vroege vormen van tumorvorming in de melkklier. Dit kan behulpzaam zijn om effectieve preventieve en/of therapeutische strategieën te ontwikkelen die een neoplastische transformatie van een hyperplastische laesie afremmen.

Tenslotte, gen expressie profielen van honden melkkliercellijnen, honden melkklier tumoren en progesteron geïnduceerde hyperplasie hebben een groot aantal genen aangetoond die verschillend tot expressie komen maar waarvan de functie niet of nauwelijks bekend is. Met behulp van de goed gekarakteriseerde cellijnen **(hoofdstuk 3)** kan meer informatie worden gekregen over hun functie in een benadering zoals beschreven in **hoofdstuk 4**, en ons begrip van de tumor biologie vergroten.

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Curriculum Vitae

Nagesha A. S. Rao was born on 22nd July 1975 in Chickmagalur district of Karnataka State, India. He obtained his BSc and MSc degrees at University of Agricultural Sciences, Bangalore, India Later he completed another MSc (Biotechnology) on April 2002 at Wageningen University, The Netherlands with a Nuffic Fellowship. He continued his education as a PhD fellow at Department of Clinical Sciences of Companion Animals, University of Utrecht, The Netherlands under supervision of Dr. Ir. Jan Mol and Prof. Jan Rothuizen. The main goal of his PhD project was to improve the molecular understanding on canine mammary tumorigenesis using canine microarray. The results of his PhD research are presented in this thesis which will be publicly defended on 13th May 2008.

List of Publications

Bhatti SF, **Rao NA**, Okkens AC, Mol JA, Duchateau L, Ducatelle R, van den Ingh TS, Tshamala M, Van Ham LM, Coryn M, Rijnberk A, and Kooistra HS. Role of progestin-induced mammary derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domest Anim Endocrinol*. 2007 Oct; 33 (3):294-312.

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Rao NA, van Wolferen ME, van den Ham R, van Leenen D, Groot Koerkamp MJ, Holstege FC and Mol JA. cDNA microarray profiles of canine mammary tumor cell lines reveal deregulated pathways pertaining to their phenotype. *Animal Genetics*. 2008 (*Accepted*)

Rao NA, Gracanin A, Slob A, Wolfswinkel J, and Mol JA. CDC2 mediated expression of RGC-32 and factor B contributes to canine mammary tumor cell proliferation. *(Submitted)*

Rao NA, Gracanin A, Mol JA. Enhanced Wnt signaling in progestin-induced and spontaneous canine mammary tumors *(Submitted)*

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Conferences and Scientific Proceedings

Nagesha A.S. Rao, Rene van den Ham, Monique van Wolferen and Jan A. Mol: Identification of differently expressed genes in canine mammary tumor cell lines using a newly developed canine specific cDNA microarray; Abstract for the 3rd International Symposium on The Molecular Biology of Breast Cancer, June 2005, Molde, Norway. (*Poster presentation*)

Nagesha Rao, Ana Gracanin and Jan Mol: Cross talk of cyclins and their kinases with the complement cascade in canine mammary cell Lines. EMBO Molecular medicine Conference on "Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression, 6th-8th June 2006, UCD, Ireland. (*Poster presentation*)

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Ana Gracanin**, Nagesha Rao,** and Jan Mol: Role of Wnt and Rac1b Signaling in canine mammary carcinogenesis. EMBO Molecular medicine Conference on "Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression, 6th-8th June 2006, UCD, Ireland. *(Poster presentation)*