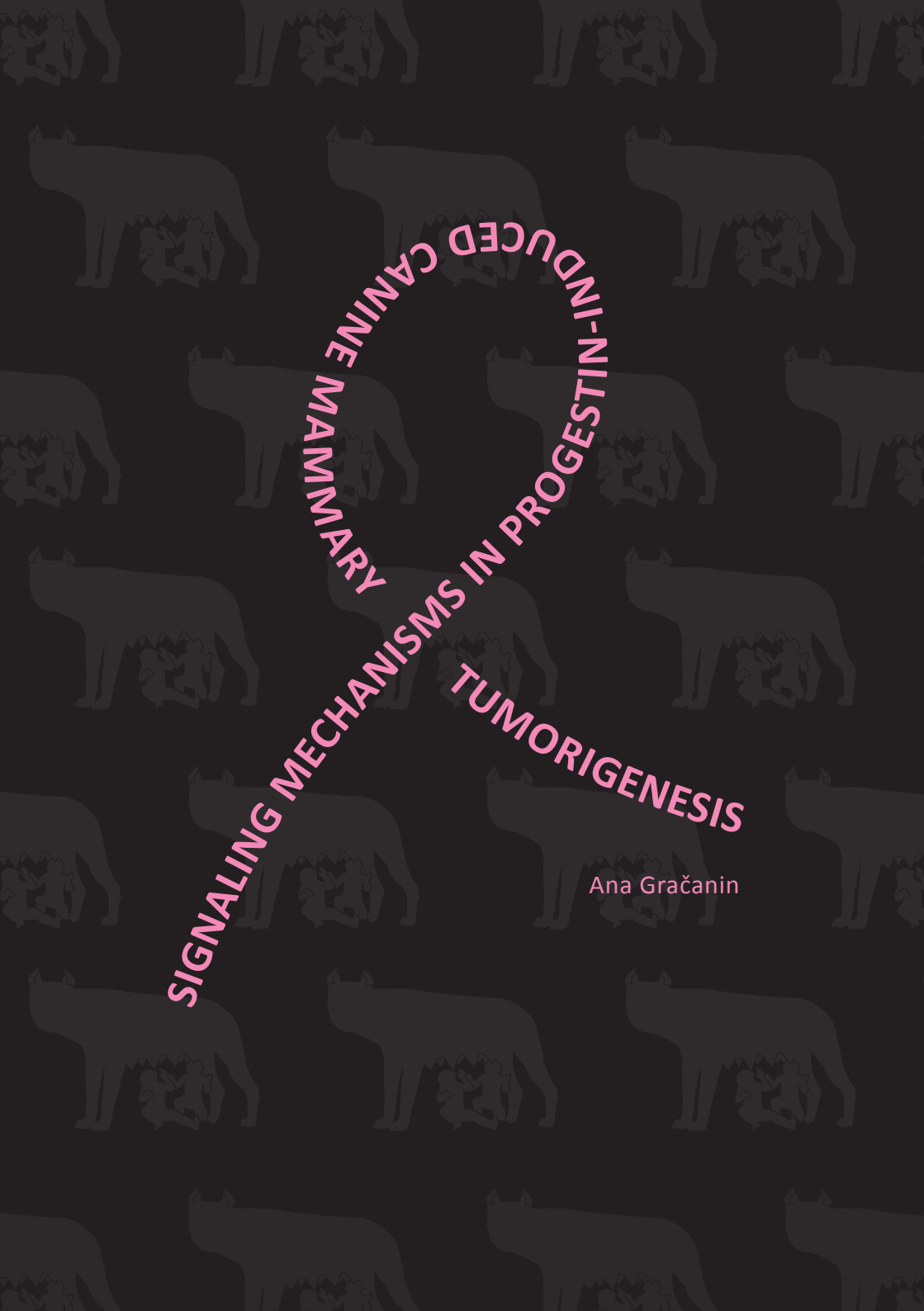
SIGNALING MECHANISMS IN PROGESTIN-INDUCED CANINE MAMMARY TUMORIGENESIS
Ana Gračanin

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Signaling mechanisms in progestin-induced canine mammary tumorigenesis

Signaalpaden in progestin-geïnduceerde honden melkklier tumorigenese

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 8 september 2015 des ochtends te 10.30 uur

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Ana Gračanin

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te Zenica, Bosnië

Promotor: Prof. dr. H.C. Clevers

Copromotor: Dr. J.A. Mol

Contents

CHAPTER 1

General Introduction

Breast cancer

Breast cancer is the most common malignancy in women, accounting for 1 out of 8 women that will be diagnosed with breast cancer within their lifetime[1,2]. Moreover, breast cancer is a very heterogeneous disease for which no defined progression model exists due to lack of clear markers. Instead different subtypes have been identified based on histopathology or molecular profiling of the tumors (Table 1). Histologically breast cancer is classified as carcinoma *in situ* or as an invasive cancer. Among invasive cancers, infiltrating ductal carcinoma is the most prevalent type accounting for 70-80% of tumors. Alternatively, molecular profiling has identified 6 breast cancer subtypes: luminal A, luminal B, normal breast-like, Her2 enriched, basal-like and claudin low. The first three subtypes are often hormone receptor (progesterone receptor (PR) and estrogen receptor (ER)) positive and account for about 70% of breast cancers[3].

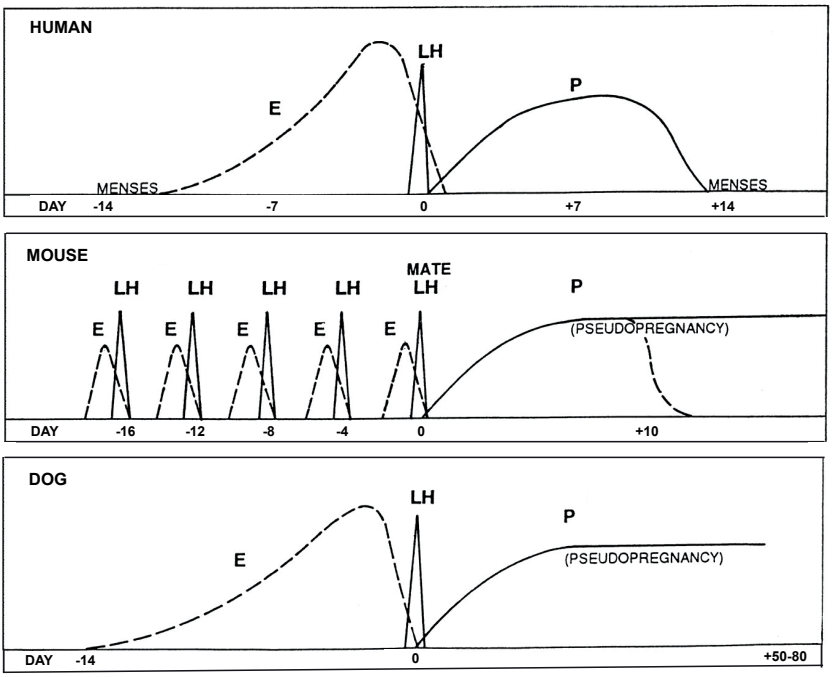
Human estrous (menstrual) cycle can be divided into 3 phases based on ovarian activity and lasts on average 28 days. Follicular phase is characterized by increasing production of estrogen, followed by ovulation induced by a surge of luteinizing hormone (LH) and a luteal phase characterized by progesterone production (Fig. 1).

Multiple protective and risk factors for breast cancer have been identified that are related to reproductive physiology, more specifically exposure to ovarian hormones (estrogen and progesterone). Early full-term pregnancy has been identified as protective[4], while early menarche, late menopause and shorter menstrual cycles were all shown to be risk factors[5]. Estrogen has been the primary therapeutic target in breast cancer and the used drugs are targeting ER signaling (i.e. tamoxifen) or the synthesis of estrogen (i.e. aromatase inhibitors). In the last years, however, progesterone and its signaling are becoming recognized as an important risk factor for breast cancer[6]. As an example, in women receiving hormone replacement therapy (HRT) a combination of estrogen and different synthetic forms of progesterone (progestins) has been identified as a risk factor[7] while HRT consisting of estrogen alone was suggested to even have a protective effect[8]. Although it is becoming accepted that progesterone is playing an important role, there is a need for understanding of signaling pathways involved in progesterone-induced mammary tumorigenesis.

Table 1 | Breast cancer classification based on histological features (left) and molecular profiling (right). Based on[3].

Histological classification		Molecular profiling
Carcinoma *in situ*	Invasive cancer	Luminal A
Ductal (DCIS) Lobular (LCIS)	Infiltrating ductal (IDC)	Luminal B
	Invasive lobuar	Normal breast-like
	Mucinous	Her2 enriched
	Tubular	Basal-like
	Medullar	Claudin low
	Papillary	

Figure 1 | Hormonal profiles during estrous cycles in human, mouse and dog. E= estrogen, LH = luteinizing hormone, P= progesterone. Adapted from[11].



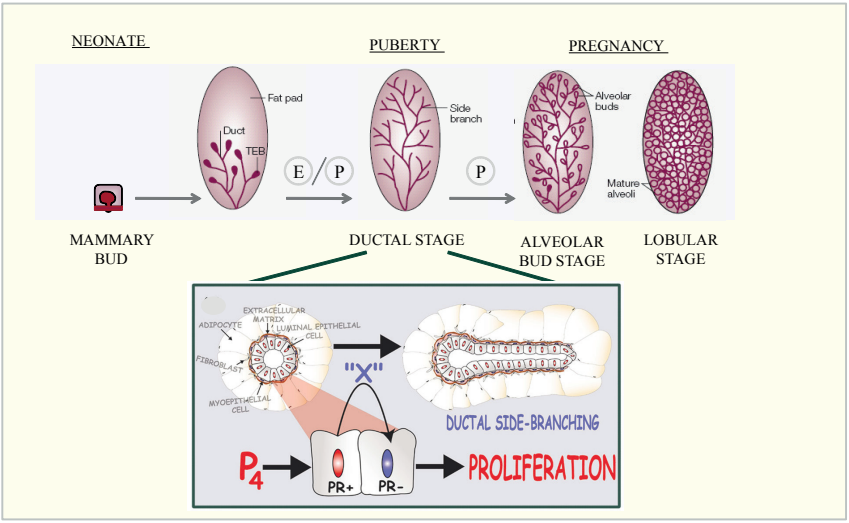
Canine progesterone-related reproductive biology and mammary tumors

Mouse models have proven very useful in deciphering the main players during mammary gland development and have been used to study the role of ovarian hormones in mammary tumorigenesis[9,10]. However, mouse models are less suitable when it comes to studying the role of progesterone in spontaneous mammary tumorigenesis. Mice namely have a very short estrous cycle allowing them to conceive every 4-5 days. This is possible because unless exposed to coital stimulation they lack the luteal phase of the cycle (Fig. 1)[11]. Furthermore, mouse tumors are, in general, hormone insensitive (with the exception of the MPA induced breast cancer model)[12] and only certain inbred strains of mice develop spontaneous mammary tumors[10]. Rats are in these aspects more suitable as a model; however, in rats metastases are relatively uncommon[6,13].

In contrast, dogs exhibit about two estrous cycles per year and are irrespective of conception exposed to obligatory (pseudo)pregnancy-associated luteal phase of 50-80 days (Fig. 1). This repeatedly prolonged exposure to high progesterone levels is thought to be the main cause of high incidence of spontaneous mammary tumors in non-spayed female dogs. In concordance, spaying before first estrous cycle greatly decreases the incidence of mammary tumors (from 26% to 0.5%)[14], while prolonged administration of exogenous progestins results in development of hyperplasia and benign mammary lesions[15]. Unlike in mice, all dog breeds develop spontaneous mammary tumors, although some variation in tumor incidence has been reported between breeds[16-18].

Classification of canine mammary tumors is currently based on histopathology, but increasing efforts are being made to classify the tumors based on molecular profiling[19]. Histologically, canine malignant tumors of epithelial origin resemble the human counterparts as carcinoma *in situ* and simple (invasive) carcinoma. In contrast to human breast cancer, however, in dogs there is a more frequent occurrence of complex carcinoma, where both epithelial and myoepithelial cells are contributing to tumor formation[20,21]. Nonetheless, comparative analysis of the role of known signaling pathways involved in mammary carcinogenesis has shown a high degree of overlap between human and canine tumors (reviewed in[22]). Moreover, sequencing of canine simple mammary carcinomas has revealed genomic aberration

Figure 2 | Progesterone in mammary gland development. Adapted from[29,31]. E=estrogen, P or P_4 = progesterone, 'x'= putative paracrine factor.



comparable to ones in human cancer, while complex carcinomas seem more to reflect epigenetic changes in breast cancer development[20].

Similarly to humans, canine mammary tumors are often positive for ER and/or PR, although percentage positive tumors vary greatly between different studies, likely due to inconsistent evaluation methods (reviewed in[23]).

Antiprogestins, such as aglepristone (RU534) are currently used to induce pregnancy termination and to treat cystic endometrial hyperplasia-pyometra in dogs[24,25]. Interestingly, aglepristone was also shown to decrease proliferation in PR-positive canine mammary carcinomas[26] and could therefore be under consideration as a neoadjuvant treatment for PR-positive canine mammary tumors[18].

Role of progesterone in normal mammary gland development and function

Initial stages of mammary gland development during embryogenesis are hormone independent and involve formation of a mammary placode

and subsequently, a mammary bud. The functional development and differentiation of the mammary gland occur mainly postnatal, under hormonal control and are coordinated with the reproductive development (Fig. 2)[27]. At the onset of puberty, estrogen and local growth factors trigger the elongation of the simple ductal tree by stimulating cell proliferation in terminal end buds (TEBs). Subsequently, fluctuating levels of progesterone stimulate the process of side-branching and development of alveolar buds. During pregnancy, in response to progesterone and prolactin, these alveolar buds can then differentiate into functional milk producing units, alveolae[28-30]. Progesterone is thought to induce these changes in the mammary gland in a paracrine manner by acting on the PR expressing ductal epithelial cells, to stimulate the expression of growth factors that evoke proliferation of the neighbouring PR negative cells[31] (Fig. 2). These putative paracrine factors involve growth hormone (GH), Wnt and receptor activator of nuclear factor kappa B ligand (RANKL)[32-35].

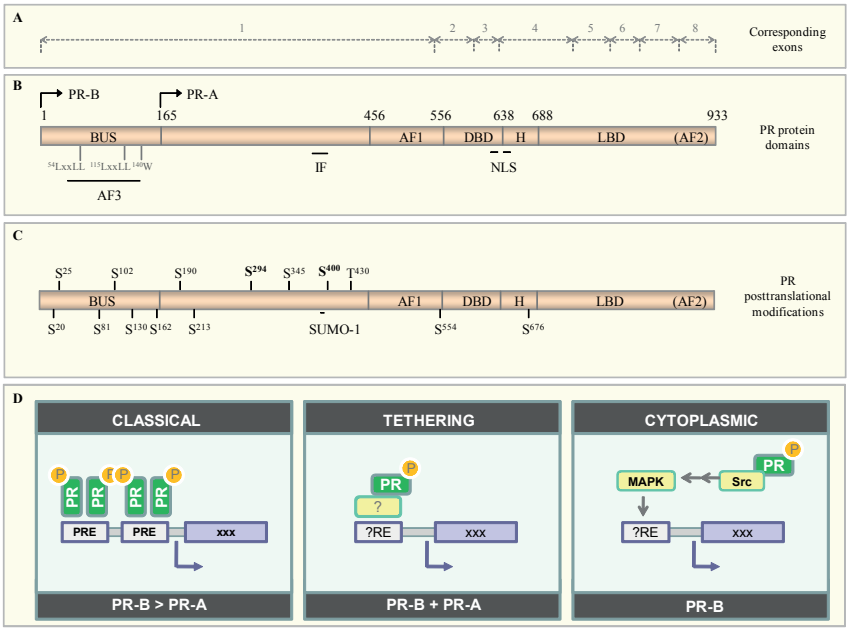
Progesterone signaling

Physiological effects of progesterone are mediated by two protein isoforms, the full-length PR-B and the N-terminally truncated PR-A that lacks the first 164 amino acids of PR-B. These two isoforms are encoded by separate transcripts that are generated by distinct estrogen-regulated promoters within the same human gene[36] (Fig. 3A and B). Based on *in vivo* expression pattern and knockout studies of individual isoform, it has been suggested that PR-B and PR-A differentially mediate reproductive functions of progesterone. This is supported by *in vitro* data, demonstrating that PR-A and PR-B in human mammary cells, regulate expression of distinct subsets of genes in a ligand-dependent and –independent manner[37,38].

Expression of PR isoforms was shown to be under hormonal control. In ovariectomized mice, PR-A expression was stimulated by estrogen and inhibited by progesterone. In contrast, PR-B levels were not affected by estrogen, but rose after prolonged treatment with progesterone or progesterone in combination with estrogen[39]. Accordingly, in mouse, PR-A is the main PR isoform expressed during pre-pubertal stages and in adult virgins, while PR-B expression increases only during pregnancy[40]. Therefore, it has been suggested that in mouse, the initial proliferative response of the mammary epithelium to progesterone, leading to side-branching is mediated by PR-A, while PR-B is needed for a proper lobuloalveolar

development during pregnancy[39]. In contrast, in normal human breast both PR-A and PR-B are coexpressed in the same cells implying species-specific regulation of the isoforms[41].

Figure 3 | Progesterone receptor structure and signaling. (A) Exons encoding different protein regions. **(B)** Overview of PR protein domains. Translation start site for the two isoforms are indicated by an arrow as well as amino acid positions bordering represented domains. BUS = PR-B specific upstream segment, AF=activation function domains, IF = inhibition function domain, DBD = DNA binding domain, H = hinge region, LBD = ligand binding domain, NLS = nuclear localization signal. **(C)** Overview of common PR posttranslational modifications. S= phosphorylated serine residue, T= phosphorylated threonine residue, SUMO-1 = sumoylation site. **(D)** Three different signaling mechanisms exerted by PR isoforms. P = progesterone, PRE = progesterone response element, ?RE = additional/other response element.



To comprehend differential physiological roles of PR-A and PR-B one should understand common and distinct structural and functional features of the individual isoform. Progesterone binding to PR induces conformational changes in the receptor and subsequent dissociation from a multi-protein chaperone complex in the cytoplasm. Upon homo/heterodimerization, ligand bound PR dimer binds to specific progesterone response elements (PREs) within the promoter of a target gene (classical signaling Fig. 3D). In order to initiate transcription this hormone activated PR recruits different coactivators that facilitate transcription initiation through protein interactions with components of general transcription machinery as well as by promoting local remodeling of chromatin at specific promoters ([42]and references therein).

Both PR isoforms share three functional domains conserved among all members of nuclear receptor superfamily[43], namely: (1) C-terminal ligand binding domain (LBD) that is also the primary mediator of receptor dimerization; (2) hinge region (HR) that contains the nuclear localization signal (NLS) of PR[44] and (3) DNA binding domain (DBD) responsible for docking the receptor to the hexanucleotide PREs within progesterone regulated gene promoters (Fig. 3B). Moreover, PR contains three activation function (AF) domains. Two of these, AF1 and AF2, are common to both PR-A and PR-B and are located upstream of DBD and within C-terminus of LBD, respectively. Although both domains regulate recruitment of coactivator proteins, AF1 in contrast to AF2 exhibits ligand-independent activity[43]. The third AF domain (AF3) is located within N-terminal PR-B specific region, also known as the PR-B specific upstream segment (BUS)[45]. The AF3 was shown to function only in the context of its homologous DBD and to synergize with AF1 and AF2 on multiple PRE-containing promoters[46]. Three motifs in BUS region (^{54}LxxLL, ^{115}LxxLL and ^{140}W) were shown to be highly conserved and were mapped as critical for AF3 function[47]. Interestingly, inhibition function (IF) domain located between the BUS and AF1 can inhibit the activity of AF1 and AF2 but not that of AF3. Due in part to this, PR-B is a much stronger activator than PR-A, under most cell and promoter contexts ([48]and references therein). Furthermore, it has been suggested that PR-A and PR-B differ in interaction with coactivator proteins. Although none of PR-interacting proteins identified thus far bind directly to the BUS region, AF3 was shown to regulate the coactivator binding to PR-B probably by affecting their binding to AF1 and AF2[46].

Postranslational modifications have been suggested to play an additional role in the transcriptional activity of steroid receptors as they may affect receptor stability, subcellular localization as well as interaction with other proteins[49]. In PR, these modifications include phosphorylation and sumoylation.

PR contains 14 known phosphorylation sites (Fig. 3D), some of which are constitutively phosphorylated (S^{81}, S^{162}, S^{190} and S^{400}) while other sites (S^{102}, S^{294} and S^{345}) become maximally phosphorylated in response to progesterone treatment[50]. PR S^{294} is considered a key site for regulation of PR location, turnover and activity as its phosphorylation increases nucleocytoplasmic shuttling of the receptor and augments PR downregulation by targeting liganded receptor for ubiquitination and subsequent proteosomal degradation[51]. PR S^{400} site is vigorously phosphorylated by mitogenic stimuli, mainly CDK2, suggesting that this site might play a role in the regulation of PR during the cell cycle[50]. Sumoylation is a dynamic process involving a covalent attachment of small ubiquitin-like modulator (SUMO) proteins like SUMO-1, to a target protein. PR, like the majority of steroid receptors targeted for sumoylation displays diminished transcriptional activity when sumoylated[52].

In addition to classical signaling, both PR isoforms can tether to other transcription factors to indirectly regulate gene expression[53]. Moreover, PR-B but not PR-A, can induce rapid cytoplasmic signaling in cells, such as MAPK signaling through its interaction with Src[54] (Fig. 3D).

Growth hormone signaling

Growth hormone (GH) is a peptide hormone closely related to prolactin (PRL) and exerts its action through GH receptor (GHR) that is presented on the cell membrane as constitutively dimerized single transmembrane protein[55]. Upon binding of GH ligands, GHR dimers are transphosphorylated by JAK2 tyrosine kinases which results in activation of multiple downstream signaling pathways, including signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, the phosphoinositide-3 kinase (PI3K) pathway and the protein kinase C (PKC) pathway[56,57]. Signaling through STAT pathway is initiated by phosphorylation of Stat proteins by JAK2. Subsequent dissociation of Stat proteins from GHR is followed by their dimerization and translocation to

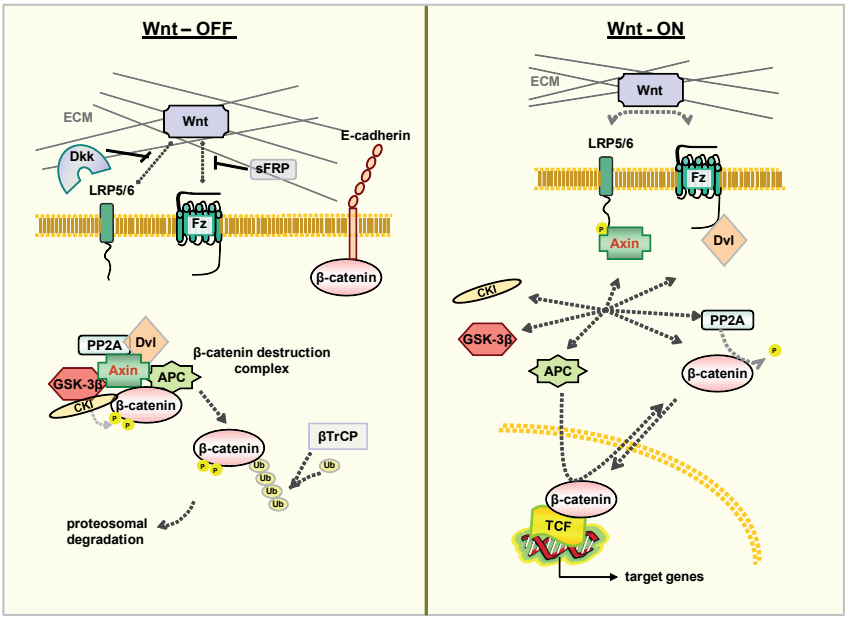
the nucleus to activate target gene expression. Activation of Stat5A and B isoforms plays an important role in the mammary gland development and results in transcriptional activation of multiple target genes including IGF-1 and serum protease inhibitor 2.1 (spi2.1)[58,59]. Luciferase construct driven by the promoter of spi2.1 gene (spi-luc) is therefore often used to assess the activity of Stat5 signaling in cells[60].

During the pubertal mammary gland development, GH signaling is involved in the ductal elongation and formation of terminal end buds (TEBs)[61]. Initially, GH effects were thought to be mediated only indirectly through induction of IGF-1, but accumulating evidence suggests that GH has also additional IGF-1 independent effects[62].

The main source of endocrine GH is the pituitary gland, but additional extrapituitary tissues can also produce GH in which the protein acts in autocrine or paracrine fashion (reviewed in[63]). Interestingly, GH expression has also been detected in the mammary gland of dogs, cats, mice and humans. This mammary GH is often found to be elevated in hyperplastic and tumorous human and canine mammary lesions where it is thought to provide proliferative signals within the tissue (independent of IGF-1) and to contribute to oncogenic transformation of mammary cells[35,64-66]. Autocrine and paracrine effects of GH in the mammary gland are further supported by the expression of GHR in normal and tumorous mammary cells[65,67,68].

In dogs, mammary GH expression was shown to be primarily under progesterone regulation. Both endogenous progesterone as well as exogenous progestins can potently induce mammary GH expression, which can even cause an excess of circulating GH resulting in acromegalic features and insulin resistance[15,69]. Moreover, in the benign mammary gland GH expression is confined only to PR positive mammary cells[70]. In a subset of malignant tumors, however, GH expression has been demonstrated also in absence of PR, suggesting a loss of progesterone control[35]. Therefore, it remains unclear whether regulation of mammary GH expression is under direct progesterone control and/or if it involves a crosstalk with other progesterone-responsive pathways.

Figure 4 | Overview of canonical Wnt signaling. Left represents a Wnt off condition, while right shows the Wnt on condition.



Canonical Wnt signaling

Wnt signaling pathway is involved in regulation of a number of processes including cell proliferation, cell polarity, differentiation and morphogenesis even from very earliest stages of embryogenesis[71]. Wnt proteins, in general, are thought to signal through four distinct pathways; the so-called canonical or Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway involving protein kinase C (PKC), the planar cell polarity pathway involving JNK and a pathway involving Protein Kinase A that functions in muscle myogenesis[72].

The most important mediator of the canonical Wnt signaling is β-catenin. In a cell lacking canonical Wnt activity (Fig. 4 left), the vast majority of β-catenin protein is bound to E-cadherin at the cell membrane where it provides a link between the actin cytoskeleton and cell-cell junctions. The so-called β-catenin destruction complex rapidly degrades the remaining β-catenin in the cytoplasm. In this complex, proteins like Axin (Axin1 and Axin2) and APC act as scaffold to bring β-catenin in association with kinases CK1 and GSK-

3β, which phosphorylate β-catenin at Ser/Thr residues. This phosphorylated β-catenin is targeted by ubiquitination complex resulting in subsequent proteosomal degradation[73]. In the case of canonical Wnt pathway activation (Fig. 4 right) through binding of Wnt ligands to Frizzled (Frz) receptors and LDL-related protein 5/6 (LRP5/6) co-receptors, the β-catenin destruction complex dissociates. As a consequence, β-catenin is dephosphorylated by protein phosphatase (PP2A), resulting in its cytoplasmic stabilization. Stabilized β-catenin is then able to translocate to the nucleus where, in association with TCF/LEF1 transcription factors it regulates expression of target genes in a tissue/cell type specific manner[74,75]. Recently, additional mediators of canonical Wnt signal have been identified that modulate stability of Frz/LRP receptor complex on the cell membrane (i.e. Lgr5 and Rspo proteins), thereby enhancing the Wnt ligand signal[76].

In order to tightly regulate the canonical pathway activity, cells also express a number of Wnt antagonists, like Dikkopf (Dkk) and secreted Frizzled related protein (sFRP) that prevent Wnt proteins from binding to Frz or LRP5/6[77,78]. In addition, activation of canonical Wnt pathway provides a negative feedback through stimulation of Axin2 expression[79].

In the mammary gland, canonical Wnt activity was shown to be essential for both embryonic and postnatal development (reviewed in[80]). During puberty and pregnancy, Wnt activity has been linked to progesterone signaling. Progesterone has been shown to promote Wnt ligand expression (especially Wnt4) and to activate the downstream signaling in both mice and dogs[34,81,82]. The relevance of this regulation is confirmed by the phenotype of conditional knockout of mammary Wnt4 that has impaired ductal side-branching[34], while overexpression of stabilized β-catenin in luminal epithelium results in precocious lobuloalveolar development, alveologenesis and neoplasia[83].

In addition to its role in normal mammary gland development, deregulation of canonical Wnt pathway is often associated with tumorigenesis[74,84]. Oncogenic properties of Wnt proteins were first evident in the mammary gland, as Wnt1 and Wnt3 ligand were initially identified as insertion sites for a mouse mammary tumor virus (MMTV) provirus[85]. Moreover, in human breast cancer, around 60% of examined clinical samples were shown to have elevated levels of nuclear and/or cytoplasmic β-catenin, implicating an active signaling[86].

In majority of human tumors, constitutive activity of the canonical Wnt pathway was shown to be a consequence of mutations in APC or β-catenin[74]. In contrast, such mutations are found only rarely in breast cancer[87]. Rather, alternative explanations for the pathway activation have been proposed for mammary tumors, including: 1) mutations in other components of the pathway, 2) overexpression of Wnt ligands[87] and other activators; 3) loss or downregulation of the antagonists, such as sFRP1[88] and 4) cross-regulation by other deregulated pathways, such as EGF, PTEN or p53 signaling[87].

Information about canonical Wnt activity in canine mammary tumors is limited. Deregulation of the pathway has been proposed based on elevated β-catenin immunostaining and pathway analysis associated with molecular profiling of normal and tumorous tissue[81,89-92]. None of the studies have, however, quantitatively assessed the activation of canonical Wnt signaling in canine mammary tumors or the underlying mechanism of its activation.

Outline of the thesis

In this thesis we focus on three unexplored aspects regarding signaling mechanisms in progestin-induced canine mammary tumorigenesis:

1) Studies focusing on expression and function of human and murine PR isoforms have revealed high degree of conservation but also species-specific features. Chapter 2, 3 and 4, therefore, first address the function and expression of canine PR isoforms. We show that dogs (and other canids) are unique among mammals in that they have multiple mutations within AF3 domain resulting in a poor PR-B related transcriptional activity. Moreover, dogs appear to have low (or no) expression of PR-B. This data is further discussed in relation to unique progesterone-related reproductive biology of canids.
2) A subset of canine mammary tumors expresses mammary GH in absence of PR, suggesting a loss of direct progesterone control and/or crossregulation by additional pathways. Similarly, canonical Wnt activity in human breast cancer was shown to be responsive to deregulation of multiple signaling pathways. In chapter 5 we, therefore, explore the possibility of direct crossregulation between GH and canonical Wnt signaling in canine mammary tumor cell lines in the absence of PR. Using luciferase reporters and gene expression analysis we observed no direct crossregulation, suggesting that the progesterone-dependent regulation of the two pathways is independent of each other.
3) Aberrant activation of canonical Wnt signaling has been suggested in canine mammary tumors based on limited immunohistochemical data and gene expression profiling. In chapter 6 we quantitatively assess the activity of canonical Wnt signaling in canine mammary tumor cell lines as well as the underlying mechanism of activation. Luciferase based TCF-reporter assay identified three groups of cell lines exhibiting high, moderate and low canonical Wnt activity. Moderate canonical Wnt activity was dependent on Wnt ligands and could be explained by overexpression of Wnt ligands and/or loss of sFRP1 antagonist. In contrast, high canonical Wnt activity was ligand-independent and was not caused by mutations in essential pathway components. Our data suggest a novel ligand-independent mechanism of canonical Wnt activation that may involve overexpression of a TCF-family member, LEF1.

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CHAPTER 2

Canid Progesterone Receptors Lack Activation Function 3 Domain Dependent Activity

Ana Gracanin[1], Monique E. van Wolferen[1], Carol A. Sartorius[2], Arjan B. Brenkman[3], Willem G. Schoonen[4] and Jan A. Mol[1]

[1] Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
[2] Department of Pathology, University of Colorado Denver, USA.
[3] Department of Metabolic Diseases and Netherlands Metabolomics Center, University Medical Center Utrecht, The Netherlands.
[4] Parklaan 16, 5345BV Oss, The Netherlands

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Abstract

Progesterone regulates multiple behavioral, physiological, and pathological aspects of female reproductive biology through its two progesterone receptors (PRs), PR-B and the truncated PR-A. PR-B is necessary for mammary gland development in mice and, compared with PR-A, overall a stronger transactivator of target genes due to an additional activation function (AF3) domain. In dogs, known for their high sensitivity to progesterone-induced mammary cancer, the PR-B function was studied. Canine PR (cPR)-B appeared to contain multiple mutations within AF3 core sequence motifs and lacks N-terminal ligand-independent posttranslational modifications. Consequently, cPR-B has a weak transactivation potential on progesterone-responsive mouse mammary tumor virus-luc and progesterone response element 2-luc reporters transiently transfected in hamster, human, or canine cells and also on known target genes FKBP5 and SGK in doxycycline-inducible, stable transfected cPR-B in canine mammary cells. The cPR-B function was restored to the level of human PR-B by the replacement of canine AF3 domain with the human one. The lack of AF3 domain-dependent transcriptional activity was unique for canids (gray wolf, red fox, and raccoon dog) and not present in closely related caniform species (brown bear, gray seal, and domestic ferret). Despite the limited transactivation potential, canids develop normal mammary glands and frequently mammary tumors. Therefore, these results question the role of PR-B in breast cancer development and may explain unique features of canid reproduction.

Introduction

The steroid hormone progesterone is crucial for normal female reproductive biology, but is also recognized as an important risk factor for breast cancer[1,2]. Progesterone activates two progesterone receptor (PR) proteins that are encoded by a single gene through usage of distinct promoters, generating PR-B and the N-terminally truncated PR-A isoform[3]. In mice PR-B is essential for proper mammary gland development, whereas PR-A is responsible for ovarian and uterine tissue responses as well as for sexual behavior[4,5]. These PR isoform-specific effects are reflected in different transcriptional activities of PR-B and PR-A on progesterone-responsive gene promoters, resulting in a markedly distinct target gene profile[6]. Although both isoforms function as a ligand-induced transcription factor, PR-B is a stronger transcriptional activator than PR-A, especially on promoters containing multiple progesterone response elements (PREs)[7,8]. These differences are due to the presence of an additional activation function (AF3) domain localized within the PR-B specific N-terminus[8]. The activity of AF3 domain in the human PR-B (hPR)-B is highly dependent on the presence of two nuclear receptor box motifs (^{55}LxxLL and ^{115}LxxLL) and a tryptophan residue (^{140}W). Mutations within these three AF3 core sequence motifs disrupt hPR-B's transcriptional activity, recruitment of co-activator proteins and change its target gene profile[9,10]. Consequently, the AF3 core sequence motifs are highly conserved across mammalian species[11,12].

Canids, including wolf- and red fox-like species, differ from other mammals in their behavioral, physiological and pathological features of reproductive biology that can directly or indirectly be related to progesterone action. These features include behavioral suppression of reproduction in subordinate females, a long progesterone-dominated phase of estrous cycle together with a frequent occurrence of pseudopregnancy and alloparenty[13,14]. Moreover, female dogs show an extreme high incidence of age-dependent, spontaneous mammary tumors and endometrial lesions, which are related to progestin exposure[15,16].

Aiming to study the underlying molecular mechanisms of mammary and endometrial sensitivity to progestins in dogs, we have previously determined the sequence of canine PR (cPR) and reported that the coding sequence of the N-terminal region has an unusually high GC content (80%)[17]. In the

present study we investigated the activation potential of the cPR-B isoform and made a comparison with other members of the canid family, caniform species and with hPR-B.

Materials and Methods

PR constructs

The coding sequence of cPR-B was obtained by gene synthesis (GenScript, USA) based on the reported sequence of canine PR-B (NM_001003074, [17]). The original nucleotide sequence was optimized prior to synthesis to lower the GC content, while encoding identical protein sequence (Suppl. Fig. S1). The cPR-B was subsequently sub-cloned from pUC57 vector into mammalian expression vector pCl-neo (Promega, The Netherlands) by AatII (New England BioLabs, Westburg, The Netherlands), EcoRI and SalI (Promega) digestion and sticky-end ligation into EcoRI and SalI digested pCl-neo vector. For pCl-neo-cPR-A, the PR-B translation start codon was removed from pCl-neo-cPR-B vector by EcoRI and BtrI digestion. To create the pCl-neo-hPR-B construct, hPR-B coding sequence was amplified from pKCRE-hPR plasmid[18] using primer set 1 (Table 1), followed by ligation into EcoRI digested pCl-neo vector. For pCl-neo-hPR-A, hPR-A coding sequence obtained by BamH1 (Promega) digestion of pKCRE-hPR was inserted into EcoRI digested pCl-neo vector. For cPR-M175A, PR-A translation initiation codon was mutated into alanine by PCR amplification of pCl-neo-cPR-B using mutation containing primer set 2 (mutated codon underlined). Human AF3 domain chimera was generated by individually amplified human AF3 and cPR-A from pCl-neo-hPR-B and pCl-neo-cPR-B, respectively, using primer sets 3 and 4. After ligation, hAF3-cPR product was inserted into EcoRI digested pCl-neo vector. Raccoon dog-, brown bear- and gray seal-AF3 domain chimera were generated likewise; only the respective AF3 domain regions were amplified from genomic DNA using primer sets 5-7. For generation of tagged human and canine AF3 domain fragments (hAF3 and cAF3, respectively), sc539 antibody epitope tag (hPR amino acids 530-580) with an added terminal stop codon was amplified using primer set 8. Sc539 tag was subsequently bluntly ligated with pCl-neo-hAF3 or pCl-neo-cAF3 fragments that were obtained through PCR amplification of pCl-neo-hPR-B or pCl-neo-cPR-B, respectively, using primer sets 9 and 10. For generation of cell lines with doxycycline-inducible expression of hPR-B, cPR-B and hAF3-cPR, coding regions

Table 1 | Primers used for the generation of various plasmid constructs and for quantitative PCR

Primer set	Construct	Forward primer	Reverse primer
1	hPR-B	5′-GTCGTCATGACTGAGCTGAAG-3′	5′-AAAGATGACATTCACTTTTTATGA-3′
2	cPR-M175A	5′-TGTCCACTGGCGAGCCGGCCAGA-3′	5′-GGCGGCCCCCTGGGAAGAGC-3′
3	hAF3	5′-CATGACTGAGCTGAAGGCAAA-3′	5′-CATGAGCGGGGACAACAC-3′
4	cAF3	5′-AGCCGGCCAGAGGGAAAG-3′	5′-CATTCATTTCTTGTGGAACAGCAG-3′
5	Raccoon dog AF3	5′-ATGACGGAGCGGACGGGAA-3′	5′-CATGAGCGGGCACACCG-3′
6	Brown bear AF3	5′-ATGACTGAGCTGAAGGCAAAA-3′	5′-CATGAGCGGGGACAACAC-3′
7	Gray seal AF3	5′-ATGACAGAGCCGAGGGCAAA-3′	5′-CATGAGCGGGGACGACAC-3′
8	Sc539 tag	5′-CTCAAGGAGGGCCTGCC-3′	5′-TCAACCATAATGACAGCCTGATG -3′
9	pCL-neo-hAF3	5′-TTCCCTTTAGTGAGGGTTAATGC-3′	5′-GAGCGGGGACAACACC-3′
10	pCl-neo-cAF3	5′-TTCCCTTTAGTGAGGGTTAATGCTTCG-3′	5′-CAGTGGACAGGCGGCCC-3′
11	AflII/EcoRI-hPR-B	5′-TCAGGTCTTAAGATGACTGAGCTG-3′	5′-GTGAACGAATTCTCACTTTTTATGA-3′
12	AflII/EcoRI-cPR-B	5′-TCAGGTCTTAAGATGACTGAACG-3′	5′-GTGAACGAATTCTCATTTCTTGTG-3′
13	AflII/EcoRI-hAF3-cPR	5′-TCAGGTCTTAAGATGACTGAGCTG-3′	5′-GTGAACGAATTCTCATTTCTTGTG-3′
14	4TO-hPR-B	5′-CCGGTCCGGGTGCAAGGTT-3′	5′-CTCGCGAGCGGGGACAACAC-3′
15	4TO-hAF3-cPR	5′-CCGGCCAGAGGGAAAGG-3′	5′-CTCGCGAGCGGGGACA-3′
16	qPCR *Rps19*	5′-CCTTCCTCAAAAAGTCTGGG-3′	5′-GTTCTCATCGTAGGGAGCAAG-3′
17	qPCR *FKBP5*	5′-GGAGAAGACCATGACATTCCA-3′	5′-AGCTCAGCATTAGGCTCGAT-3′
18	qPCR *SGK*	5′-TGGGCCTGAACGACTTTATT-3′	5′-GAGGGGTTGGCATTCATAAG-3′

from pCl-neo-hPR-B, pCl-neo-cPR-M175A and pCl-neo-hAF3-cPR were subcloned into AflII/EcoRI digested pcDNA-4TO by PCR amplification. During amplification flanking AflII and EcoRI restriction sites were added (underlined) using primer sets 11-13. In 4TO-hPR-B and 4TO-hAF3-cPR the PR-A translation initiation codons were subsequently mutated into alanine (underlined) using primer sets 14 and 15. All oligonucleotides were ordered from Eurogentec (The Netherlands) and all amplifications were performed using Phusion Hot Start Polymerase (Finnzymes, Bioke, The Netherlands) in the provided GC buffer supplemented with 3 % DMSO. Construct sequences were routinely confirmed by sequencing.

Cell lines and culture

CHO-K1 cells[18] were cultured on DMEM:F12 (Invitrogen, The Netherlands) supplemented with 10 % FBS (FBS Gold, PAA). T47D-Y cells[19] were maintained on MEM (Invitrogen) supplemented with NEAA (Invitrogen), 10 nM insulin and 5 % FBS. Canine mammary cell lines CNMp and CNMm

cells were kindly provided by Prof. N. Sasaki[20] and were maintained on DMEM:F12 supplemented with 10 % FBS. For generation of cell lines with stable inducible expression of hPR-B, cPR-B or hAF3-cPR, T-REx system (Invitrogen) was used. For this purpose CNMm cells were stably transfected with pcDNA6/TR and the single cell clone with the highest Tet-R expression (CNMm-6TR) (data not shown) was subsequently stably transfected with individual PR-B constructs. To ensure controlled transgene expression, the three cell lines were cultured on DMEM:F12 supplemented with 10 % tetracycline negative FBS (PAA), 3 µg/ml blasticidin (Invitrogen) and 400 µg/ml zeocin (Invitrogen).

Luciferase assays

Cells were seeded in 24-well plates (Primaria) at 70000 (CHO-K1 and CNMp) or 80000 (T47D-Y) cells/well, 48 h before transfection. Transfections were performed in absence of FBS using, per well, 2 µl Lipofectamine 2000 (Invitrogen), 800 ng MMTV-luc[18] or PRE2-luc[10], 0.3 ng human β-actin-promoter renilla[21] as an internal control and 10 ng PR constructs or empty pCl-neo vector. After 5 h the medium was changed to DMEM:F12 or MEM supplemented with 5 % charcoal-stripped FBS (PAA). Cells were left to recover for 24 h, followed by a 24 h treatment with 20 nM P. The firefly and renilla luciferase activities were subsequently quantified using Dual luciferase assay system (Promega) in a Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium). Differences in transactivation potential were statistically assessed using unpaired, 2-tailed student's T-test.

Western blotting

For protein expression analysis, CHO-K1 cells were seeded in 6-well plates (Primaria) at 200,000 cells/well, 48h before transfection. Transfections were performed with 10 µl Lipofectamine 2000 and 1 µg PR constructs per well, as described above. Protein was harvested 24 h after transfection using RIPA buffer (6.5 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl, pH 7.4; 1 % SDS (v/v), 1 % Igepal (v/v), 0.5 % Na-deoxycholate (w/v), 1 mM PMSF, 1 mM Na-orthovanadate and 1 µg/ml aprotinin). CNMm-hPR-B, -cPR-B and -hAF3-cPR cells were seeded in 6-well plates (Primaria) at 400,000 cells/well 24h prior to 24h treatment with 1 µg/ml DOX (Sigma). Total cell lysates were obtained using RIPA buffer. Protein concentration was determined using BioRad Dc Protein Assay (BioRad). 10 µg of total cell lysates was subjected to SDS-PAGE, and analyzed by Western blot using

primary antibody against PR (sc-538 or sc-539, Santa Cruz biotechnology) or Pan actin (NeoMarkers/Labvision, UK) and the secondary goat anti rabbit or goat anti mouse HRP-conjugated antibody (HAF008 or HAF007, respectively, R&D Systems). HRP was visualized using AdvanceTM Enhanced chemiluminescence (ECL, Amersham, GE Healthcare) and analyzed using GelDoc2000 (BioRad, The Netherlands).

Real time quantitative RT-PCR

Total RNA from cell lines was isolated and treated with DNase using RNeasy mini kit (Qiagen, The Netherlands) according to manufacturer's protocol. cDNA synthesis was performed using iScript kit (BioRad). Specific primer sets were used to amplify gene products (Table 1). The reactions were performed and measured using BioRad MyIQ detection system (BioRad) with SYBR green fluorophore. Relative target gene expression was normalized to that of the reference gene *Rps19* and relative induction of gene expression was statistically assessed using paired, 2-tailed student's T-test.

Immunoprecipitation and mass spectrometry (MS)

For immunoprecipitation, CHO-K1 cells were seeded in 25 cm^2 tissue culture flasks (Cellstar, Greiner bio-one) and transiently transfected with 8 µg cPR-B construct. Cell lysate was harvested 24 h after transfection using RIPA buffer, followed by immunoprecipitation in a reaction with 1 mg total cell lysate, 10 µl sc-538 antibody and 20 µl protein A-agarose beads (sc-2001, Santa Cruz biotechnology). Immunoprecipitated proteins were separated by 8% SDS-PAGE and stained with SimplyBlue Safestain (Invitrogen). Bands were excised from gel, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Roche) as described[22]. Samples were subjected to nanoflow LC (Eksigent) using C18 reverse phase trap columns (Synergi™ 4µm Hydro-RP 80 Å, Phenomenex; column dimensions 2cm x 100µm, packed in-house) and subsequently separated on C18 analytical columns (ReproSil-Pur 120 C18-AQ, 5 µm, Dr. Maisch GmbH; column dimensions, 20cm x 50µm; packed in-house) using a linear gradient from 0 to 40% buffer B (buffer A = 5% (v/v) acetonitrile, 0.1% formic acid (v/v); buffer B = 95% (v/v) acetonitrile, 0.1% formic acid (v/v)) in 60 min at a constant flow rate of 150 nl/min. Column eluate was directly coupled to a LTQ-Orbitrap-XL mass spectrometer (Thermo Scientific) operating in positive mode, using lock spray internal calibration. Data were processed and subjected to database searches using Proteome Discoverer software (Thermo Scientific) against

non-redundant NCBI database, with a 10 ppm mass tolerance of precursor and 0.5Da for the fragment ion.

Genomic DNA and PR sequencing

Genomic DNA of raccoon dog and red fox were kindly provided by Prof. Marek Switonski (Poznan University, Poland). Genomic DNA of brown bear, gray seal, domestic ferret and gray wolf were obtained from the internal DNA-collection of the Veterinary Faculty (Utrecht University). AF3 domain regions of the respective caniform species were amplified with primers developed for the canine sequence forward 5'-AGGAGAGGGGAGTCCCGGTCGTCAT-3' and reverse 5'-CCGCAGCCGTCGCCAGCCTTG-3' using Phusion Hot Start Polymerase in the provided GC buffer supplemented with 3 % DMSO. These sequence data have been submitted to the GenBank database under following accession numbers: JN182650 (red fox), JF896481 (brown bear), JF896482 (gray seal), JF896483 (raccoon dog), JQ337865 (domesticated ferret) and JQ337866 (gray wolf). The sequences were aligned using ClustalW[23].

Results

Because N-terminal region of cPR-B has been shown to have an unusually high GC content, we generated a multispecies alignment from all mammalian PR N-terminal protein sequences deposited in GenBank and Ensembl databases (Table 2). The region showed overall low conservation, except for the PR-B specific AF3 core sequence motifs. The two LxxLL motifs and the tryptophan residue were highly conserved in all mammals except the cPR sequence that contained multiple amino acid replacements within all three AF3 core sequence motifs as well as a unique ten amino acid polyalanine repeat (Fig. 1).

To address the functionality of the canine AF3 domain, constructs encoding human PR (hPR) and cPR isoforms were transiently transfected into CHO-K1 cells (Fig. 2A). Both canine PR-A (cPR-A) and human PR-A (hPR-A) were expressed as 82- and 94-kDa proteins. When stably expressed in CHO-K1 cells, both 82-kDa and 94-kDa proteins undergo ligand-induced phosphorylation and downregulation (data not shown). Even though cPR-B and hPR-B have a comparable predicted molecular weight, transfection of cPR-B construct

resulted in expression of a single 108-kDa protein, compared to 116-kDa of the hPR-B. The lower apparent molecular weight of cPR-B was not a result of N-terminal protein cleavage as shown by MS (Fig. 2B). Transfection of tagged cAF3 lacked the upshifted multiband pattern that in human AF3 domain results from multiple ligand-independent phosphorylations (Fig. 2C)[8].

Table 2 | PR sequences used for the multiple species alignment

Code	Common name	Scientific name	GenBank/ENS accession number
Cf	Dog	*Canis familiaris*	NP_001003074.1
Mm	Mouse	*Mus musculus*	NP_032855.2
Rn	Rat	*Rattus norvegicus*	NP_074038.1
Oc	Rabbit	*Oryctolagus cuniculus*	NP_001075736.1
Ec	Horse	*Equus caballus*	XP_001498544.2
La	African elephant	*Loxodonta africana*	ABE73092.1
Dn	Nine-banded armadillo	*Dasypus novemcinctus*	ABE73090.1
Dd	Dugong	*Dugong dugon*	ABE73091.1
Bt	Cattle	*Bos taurus*	XP_583951.4
Bs	Three-toed sloth	*Bradypus sp. DEW-2006*	ABE73089.1
Ss	Pig	*Sus scrofa*	NP_001159960.1
Hs	Human	*Homo sapiens*	NP_000917.3
Tb	Western tarsier	*Tarsius bancanus*	ABE73094.1
Pt	Common chimpanzee	*Homo (Pan) troglodytes*	NP_001129085.1
Pp	Bonobo	*Homo (Pan) paniscus*	ABB72141.1
Ggo	*Gorilla*	*Gorilla gorilla*	ABB72142.1
Ppy	*Orangutan*	*Pongo pygmaeus*	ABB72143.1
Hl	*White handed gibbon*	*Hylobates lar*	ABB72144.1
Ms	*Barbary macaque*	*Macaca sylvanus*	ABB72145.1
Pa	*Olive baboon*	*Papio anubis*	NP_001158062.1
Ca	*African green monkey*	*Chlorocebus aethiops*	ABB72147.1
Cg	*Black-and-white colobus monkey*	*Colobus guereza*	ABB72148.1
To	*Dusky leaf-eating monkey*	*Trachypithecus obscurus*	ABB72149.1
Cap	*Tufted capuchin monkey*	*Cebus apella*	ABE73084.1
Pi	*Gray saki monkey*	*Pithecia irrorata*	ABE73085.1
Ap	*Black spider monkey*	*Ateles paniscus*	ABE73086.1
Ocr	*Thick-tailed bushbaby*	*Otolemur crassicaudatus*	ABE73087.1
Mc	*Giant mouse-lemur*	*Mirza coquereli*	ABE73088.1
Bh	*Hoolock gibbon*	*Bunopithecus hoolock*	AAY84554.1
Tp	*Phayre's leaf monkey*	*Trachypithecus phayrei*	AAY84552.1

Figure 1 | Multispecies alignment for PR-B specific N terminus based on the sequences from Table 2. Given on top is the consensus sequence. The two LxxLL and W core sequence motifs for AF3 function are shaded yellow. In blue the canine polyalanine insert.

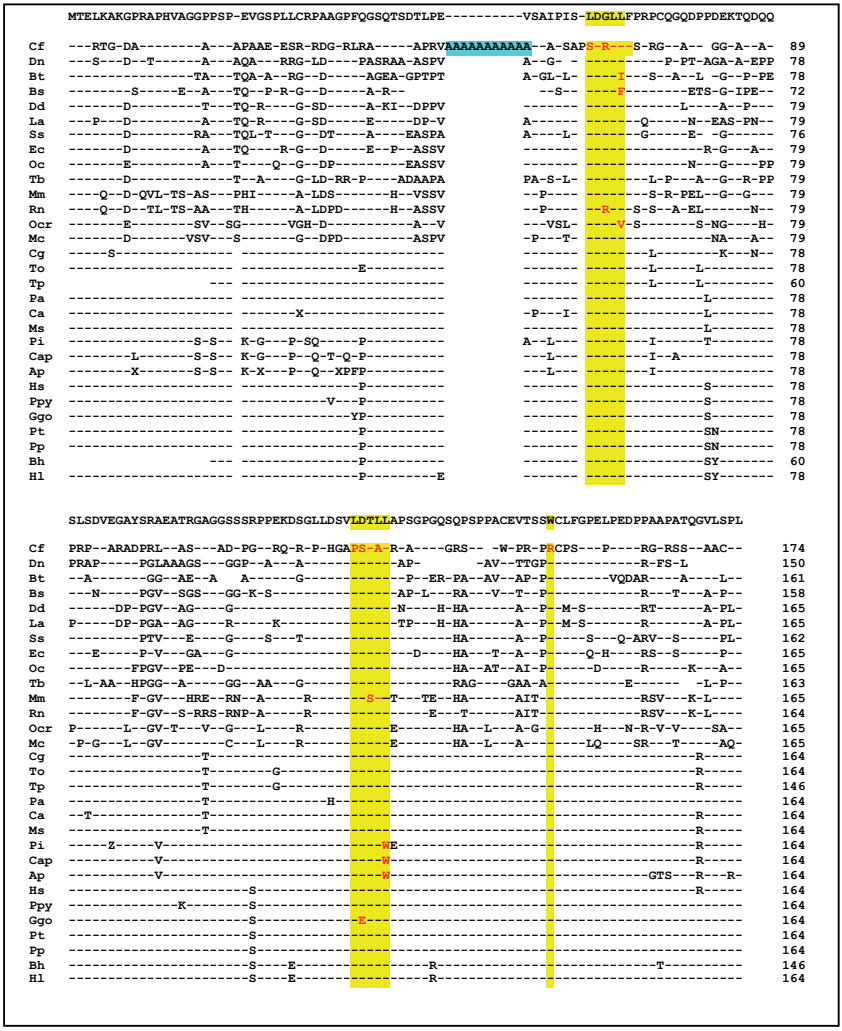
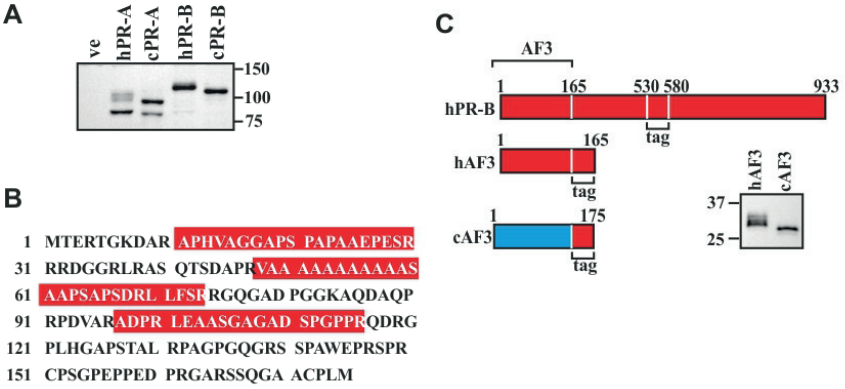
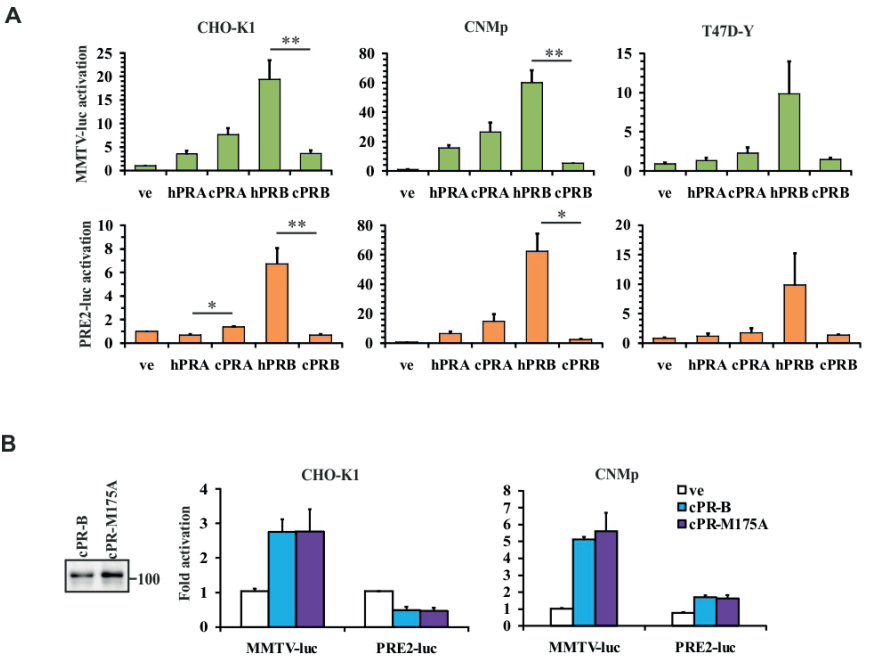


Figure 2 | cPR-B has a lower apparent molecular weight compared to hPR-B, not due to cleavage within cAF3, but partially due to lack of ligand-independent post-translational modifications. **(A)** Total cell lysate of CHO-K1 cells transfected with hPR-A, cPR-A, hPR-B, PR-B or an empty vector (ve) and immunoblotted with PR antibody. **(B)** Mass spectrometry analysis of immunoprecipitated cPR-B identified peptides (red blocks) that were scattered across the whole AF3 domain region. **(C)** Upon transient transfection into CHO-K1 cells, tagged cAF3 lacked the upshifted multiband pattern.



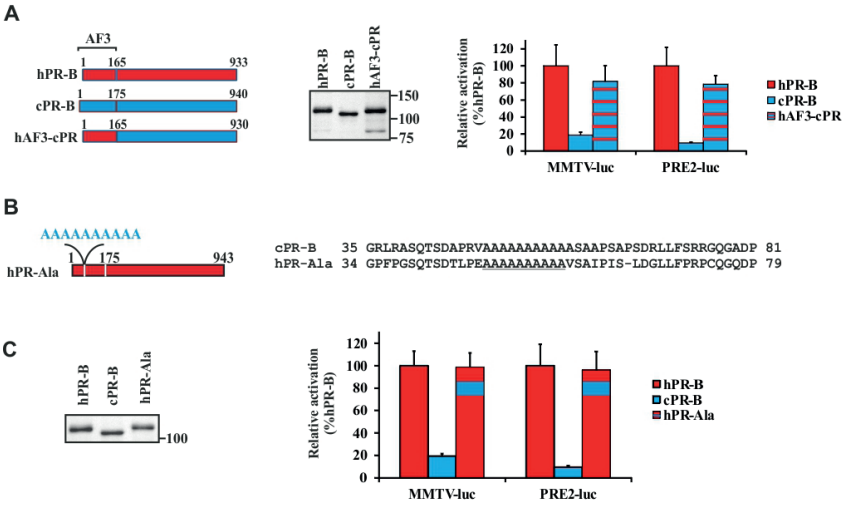
The AF3 domain is important for the transactivation of promoters containing multiple tandem PREs[9], among others, MMTV-luc and PRE2-luc. MMTV-luc contains a natural MMTV promoter with a palindromic PRE and additional three PRE half-sites, whereas PRE2-luc uses a tyrosine amino transferase promoter with two tandem palindromic PREs[10,18]. Upon transient cotransfection of cPR and hPR isoforms into hamster, human and canine cell lines (CHO-K1, T47D-Y and CNMp, respectively), progesterone-dependent transactivation of both MMTV-luc and PRE2-luc reporters by cPR-A was comparable with or slightly higher than that of hPR-A (Fig. 3A). In contrast, cPR-B was a much weaker transactivator compared to hPR-B and even PR-As, on both luciferase reporters. The transcriptional activity of cPR-B was not the result of residual expression of cPR-A, as a point mutation within the PR-A translation initiation codon of the cPR-B construct had no effect on cPR-B's transcriptional activity (Fig. 3B). To assess if the attenuated transcriptional activity of cPR-B is caused by changes in the N

Figure 3 | cPR-B has attenuated transactivation potential. **(A)** Relative induction (± s.e.m.) of MMTV-luc and PRE2-luc in transiently transfected CHO-K1, CNMm and T47D-Y cells upon 20 nM progesterone treatment. *p<0.05, **p<0.01. **(B)** PR immunoblot of transiently transfected CHO-K1 cells and progesterone-dependent transactivation potential of cPR-B, cPR-M175A and the empty vector (ve) in CHO-K1 and CNMp cells.



terminus, cAF3 domain region was replaced by the human domain (hAF3-cPR). Upon transient transfection into CHO-K1 cells, the transactivation potential of hAF3-cPR chimera resembled that of hPR-B (Fig. 4A). In addition to mutations in AF3 core sequence motifs, cPR-B's AF3 domain contains a unique repeat of ten alanines (Fig. 1). Expanded polyalanine repeats have been shown to lead to protein dysfunction[24] prompting to test the causative link between the polyalanine repeat within cPR-B's AF3 domain region and the attenuated transcriptional activity. For this purpose, a canine-like polyalanine repeat was inserted into the corresponding region of hPR-B (Fig. 4B) and the mutant (hPR-Ala) was tested for its transactivation potential

Figure 4 | Low transactivation potential of cPR-B is caused by the changes within AF3 domain independent of the polyalanine repeat. **(A)** Illustration of hAF3-cPR chimera with amino acids indicated above each construct, together with an immunoblot and the induction of MMTV-luc and PRE2-luc upon 20 nM progesterone treatment in transiently transfected CHO-K1 cells. **(B)** Illustration of the hPR-Ala mutant (left) and a partial sequence alignment of cPR-B and hPR-Ala sequence (right). Inserted polyalanine repeat is underlined. **(C)** Immunoblot of hPR-B, cPR-B and hPR-Ala and the induction of MMTV-luc and PRE2-luc upon 20nM progesterone treatment in transiently transfected CHO-K1 cells.



on MMTV-luc and PRE2-luc constructs. In this context, the presence of polyalanine repeat did not affect the function of hPR-B (Fig. 4C).

To assess whether the attenuated transactivation potential of cPR-B is also reflected in regulation of endogenous PRE-containing target gene promoters, we generated canine mammary cell lines stably expressing hPR-B, cPR-B or hAF3-cPR under DOX-inducible promoters (Fig. 5A). In concordance with luciferase assays, cPR-B (in contrast to hPR-B and hAF3-cPR) had no or only a weak effect on the expression of FKBP5 and SGK (Fig. 5B).

Figure 5 | cPR-B has an attenuated ability to regulate endogenous PRE-containing target genes. **(A)** PR immunoblot of canine mammary cell lines expressing hPR-B, cPR-B or hAF-cPR under DOX-inducible promoter. Antibody against actin was used as loading control. **(B)** Induction of *FKBP5* and *SGK* mRNA expression without (-P) or upon (+P) 24h, 20 nM progesterone treatment in presence of DOX. Expression was normalized with *Rps19* as a reference gene. *p<0.05, **p<0.01.

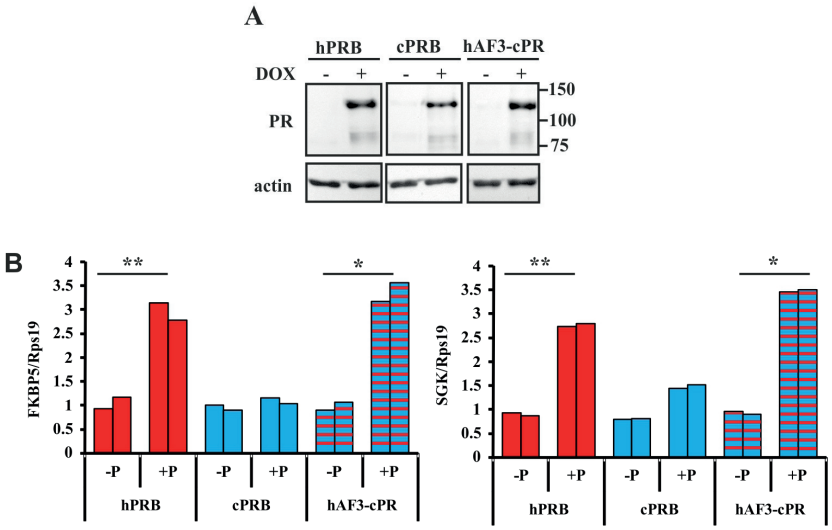
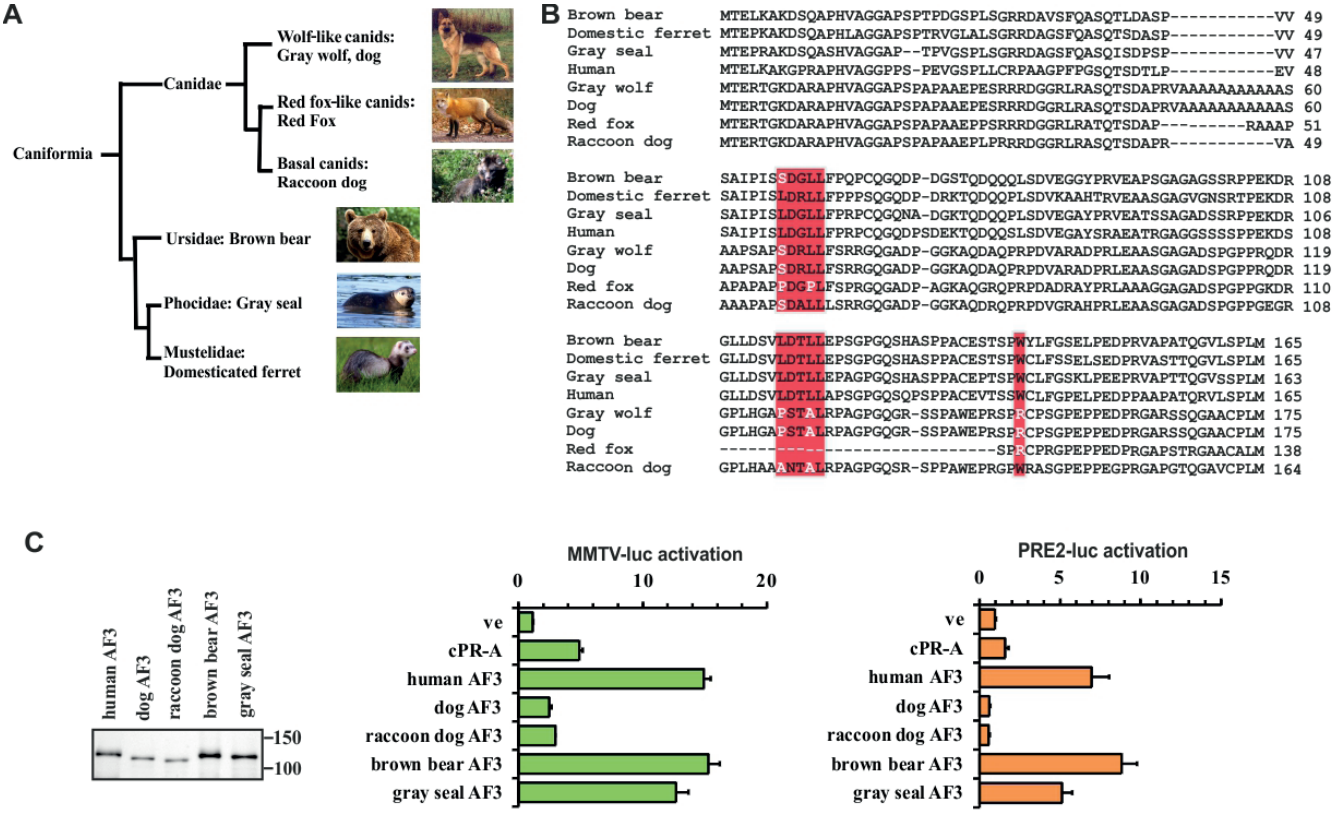


Figure 6 | Loss of AF3 domain activity is unique for canids. **(A)** Phylogeny of the analyzed caniform species (according to[33]). **(B)** Protein sequence alignment of human, canine and additionally sequenced caniform AF3 domain regions. AF3 core sequence motifs are indicated in *red blocks* and the amino acid replacements deviating from the optimal motif are depicted in *white*. **(C)** PR immunoblot of AF3 domain-cPR-A chimera and their progesterone-dependent induction of MMTV-luc and PRE2-luc. Empty vector (ve) is used as a negative control.

Figure 6


A
Caniformia
Canidae
Wolf-like canids:
Gray wolf, dog
Red fox-like canids:
Red Fox
Basal canids:
Raccoon dog
Ursidae: Brown bear
Phocidae: Gray seal
Mustelidae:
Domesticated ferret
B
Brown bear MTELKAKDSQAPHVAGGAPSPTPDGSPLSGRRDAVSFQASQTLDASP----------VV 49
Domestic ferret MTEPKAKDSQAPHLAGGAPSPTRVGLALSGRRDAGSFQASQTSDASP----------VV 49
Gray seal MTEPRAKDSQASHVAGGAP--TPVGSPLSGRRDAGSFQASQISDPSP----------VV 47
Human MTELKAKGPRAPHVAGGPPS-PEVGSPLLCRPAAGPFFPGSQTSDTLP----------EV 48
Gray wolf MTERTGKDARAPHVAGGAPSPAPAAEPESRRRDGGRLRASQTSDAPRVAAAAAAAAAAS 60
Dog MTERTGKDARAPHVAGGAPSPAPAAEPESRRRDGGRLRASQTSDAPRVAAAAAAAAAAS 60
Red fox MTERTGKDARAPHVAGGAPSPAPAAEPPSRRRDGGRLRATQTSDAP---------RAAAP 51
Raccoon dog MTERTGKDARAPHVAGGAPSPAPAAEPLPRRRDGGRLRASQTSDAPR----------VA 49
Brown bear SAIPISSDGLLFPQPCQGQDP-DGSTQDQQQLSDVEGGYPRVEAPSGAGAGSSRPPEKDR 108
Domestic ferret SAIPISLDRLLFPPPSQGQDP-DRKTQDQQPLSDVKAAHTRVEAASGAGVGNSRTPEKDR 108
Gray seal SAIPISLDGLLFPRPCQGQNA-DGKTQDQQPLSDVEGAYPRVEATSSAGADSSRPPEKDR 106
Human SAIPISLDGLLFPRPCQGQDPSDEKTQDQQSLSDVEGAYSRAEATRGAGGSSSSPPEKDS 108
Gray wolf AAPSAPSDRLLFSRRGQGADP-GGKAQDAQPRPDVARADPRLEAASGAGADSPGPPRQDR 119
Dog AAPSAPSDRLLFSRRGQGADP-GGKAQDAQPRPDVARADPRLEAASGAGADSPGPPRQDR 119
Red fox APAPAPPDGPLFSPRGQGADP-AGKAQGRQPRPDADRAYPRLAAAGGAGADSPGPPGKDR 110
Raccoon dog AAAPAPSDALLLSRRGQGADP-GGKAQDRQPRPDVGRAHPRLEAASGAGADSPGPPGEGR 108
Brown bear GLLDSVLDTLLEPSGPGQSHASPPACESTSPWYLFGSELPEDPRVAPATQGVLSPLM 165
Domestic ferret GLLDSVLDTLLEPSGPGQSHASPPACESTSPWCLFSSELSEDPRVASTTQGVLSPLM 165
Gray seal GLLDSVLDTLLEPAGPGQSHASPPACEPTSPWCLFGSKLPEEPRVAPTTQGVSSPLM 163
Human GLLDSVLDTLLAPSGPGQSQPSPPACEVTSSWCLFGPELPEDPPAAPATQRVLSPLM 165
Gray wolf GPLHGAPSTALRPAGPGQGR-SSPAWEPRSPRCPSGPEPPEDPRGARSSQGAACPLM 175
Dog GPLHGAPSTALRPAGPGQGR-SSPAWEPRSPRCPSGPEPPEDPRGARSSQGAACPLM 175
Red fox ----------SPRCPRGPEPPEDPRGAPSTRGAACALM 138
Raccoon dog GPLHAAANTALRPAGPGQSR-SPPAWEPRGPWRASGPEPPEGPRGAPGTQGAVCPLM 164
C
human AF3
dog AF3
raccoon dog AF3
brown bear AF3
gray seal AF3
150
100
MMTV-luc activation
0
10
20
ve
cPR-A
human AF3
dog AF3
raccoon dog AF3
brown bear AF3
gray seal AF3
PRE2-luc activation
0
5
10
15
ve
cPR-A
human AF3
dog AF3
raccoon dog AF3
brown bear AF3
gray seal AF3

The sequence of cAF3 domain was confirmed using genomic DNA from several dog breeds and appears to be breed-nonspecific (data not shown). With the peculiar progesterone-related reproductive biology of dogs and other canids in mind, we hypothesized that the inactivation AF3 domain may be conserved in and restricted to other members of the Canidae family (*e.g.* gray wolf, red fox and raccoon dog) (Fig. 6A). Sequencing of AF3 domain regions from genomic DNA of representative Canidae species revealed that the accumulation of mutations within AF3 core sequence motifs is indeed conserved in other canids (Fig. 6B). The gray wolf sequence was identical to the dog, whereas the red fox sequence contained multiple amino acid substitutions within AF3 core sequence motifs and even a small deletion including the second LxxLL motif. Among analyzed canids, AF3 core sequence motifs of raccoon dog contained the least number of changes. In contrast, in species closely related to canids, such as brown bear (Ursidae family), gray seal (Phocidae family) and domestic ferret (Mustelidae family), AF3 core sequence motifs remained conserved with an exception of brown bear having a single amino acid substitution within the first LxxLL motif. To evaluate the functional properties of the sequenced AF3 domains, brown bear-, gray seal- and raccoon dog-AF3 domain regions were coupled to cPR-A, generating chimeric PR-Bs. These were transiently transfected into CHO-K1 cells and tested for their transactivation potential on MMTV-luc and PRE2-luc reporters. In agreement with the sequencing results, transactivation potential of the bear- and seal-chimera was similar to that of hAF3-cPR, whereas the raccoon dog-chimera resembled that of cPR-B (Fig. 6C).

Discussion

Expression of the cPR-A in CHO-K1 cells revealed 82- and 94-kDa PR-A proteins, comparable to the previously reported data for human PR-A, both *in vitro*[25-27] and *in vivo*[28]. In addition, the transactivation potential on the MMTV-luc and PRE2-luc reporters showed that canine and hPR-A function in a comparable fashion. This is obviously not the case for the cPR-B molecule. The lower molecular weight of cPR-B compared to hPR-B was not due to N-terminal protein cleavage, but it can partially be explained by the lack of ligand-independent posttranslational modifications within cPR-B's AF3 domain. Canid-specific mutations in AF3 core sequence motifs are most

likely not causative for the lower molecular weight, because the mutation of all three motifs within hPR-B did not affect its apparent molecular weight[9].

The cPR-B showed a low to absent transactivation potential on MMTV-luc and PRE2-luc reporters, which could be rescued by hAF3, confirming a functional defect within cAF3. Next to the reporter constructs also two progesterone target genes, *FKBP5* and *SGK*, with PRE-containing promoter sequences[29,30] were not transactivated by cPR-B. It is our conclusion that cPR-B clearly lacks the AF3 domain function and has consequently lost its transactivation potential on classical progesterone target genes.

The lack of classical PR-B signaling is found also in other members of the Canidae family, including gray wolf, red fox and raccoon dog, but not in the related families of the suborder caniformia. The least mutations were found in the AF3 sequence of the raccoon dog suggesting that mutations in the second LxxLL motif play a major role in the loss of the transactivation potential.

Altogether, these findings have multidisciplinary implications. 1) In mice, PR-B expression is essential for proper mammary gland development[4]. Normal mammary gland development in dogs[31] implies that there are species-specific differences in the requirement of PR-B in the mammary gland or that PR-B function in this tissue does not involve the classical PRE-directed signaling. In this regard it is interesting that hPR-B (but not PR-A) can also signal directly through cytoplasmic interaction with c-Src[32]. The sequence motifs necessary for this interaction have remained conserved in cPR (data not shown). 2) In addition, PR signaling has been implicated in breast cancer development[2,32]. Due to the high sensitivity of dogs to progesterone in terms of mammary tumors, the unique alteration of cPR-B function is very intriguing and may provide insight into the role of cPR-B and/or hPR-B in breast cancer development. 3) Although the ultimate causation of the canid-specific reproductive biology can often be deducted from their social organization[13], there is so far little insight into the underlying proximate mechanisms. Loss of AF3 domain function in canid PR-B may be highly relevant in this context. Because most of 36 canid species[33] are currently endangered, understanding the molecular basis of their unusual reproductive biology may improve the assisted breeding technology and thereby conservation of species.

Acknowledgments

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CHAPTER 3

Marginal activity of progesterone receptor B (PR-B) in dogs but high incidence of mammary cancer

Ana Gracanin[1]*, Fabiana A. Voorwald[2]*, Monique van Wolferen[1], Elpetra Timmermans-Sprang[1], Jan A. Mol[1]

[1] Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[2] FCAV, UNESP - Univ Estadual Paulista, Jaboticabal, SP, Brazil

* Both authors contributed equally to this manuscript

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Abstract

Progesterone plays an important role in the normal development and carcinogenesis of the mammary gland. *In vitro* studies have shown that the canine progesterone receptor B (cPR-B), which is essential for mammary development in the mouse, does not transactivate reporter constructs containing progesterone response elements. Therefore the question was raised whether the cPR-B was completely devoid of transactivation potential of endogenous progesterone regulated genes.

Canine mammary cell lines expressing doxycycline-inducible cPR-B, human PR-B or a chimera in which the canine B-upstream segment (BUS) was replaced by a human BUS were treated for 24h with doxycycline, progesterone or a combination of the two. The expression profiling was subsequently performed using a dog-specific microarray and miRNA primers.

Incubation of stably transfected cell lines with doxycycline or progesterone alone, did not change expression of any endogenous gene. Expression of activated human PR-B or the chimera of human BUS with the canine PR resulted in differential expression of >500 genes whereas the activated cPR-B regulated only a subset of 40 genes and to a limited extent. The relevance of the marginal transactivation potential or the consequence of a lack of cPR-B function for the carcinogenesis of mammary gland tumors is discussed.

Introduction

Breast cancer is the most prevalent malignant disease in women[1]. About two/third of human breast cancers are steroid hormone receptor (ER/PR) positive and treated with combinations of selective estrogen receptor modulators (SERMs), GnRH agonists and/or aromatase inhibitors[2-4], alone or in combination with third-generation cytotoxic or biological therapies[5, 6]. A significant part of women treated by adjuvant hormonal therapies become ultimately therapy resistant[7, 8] showing a relapse and development of distant metastasis.

From studies early this century on hormone-replacement therapies (HRT) it is concluded that the synthetic progestin medroxyprogesterone acetate (MPA), rather than estrogens given as conjugated equine estrogens, are the major cause of enhanced breast cancer incidence in women on HRT[9]. Since then a numerous studies reported on the effects of various synthetic progestins and the result of cessation of HRT on breast cancer incidence. Ten years after the first report by Chlebowski *et al*, it was stated that progesterone signaling in breast cancer was neglected for too long and is now coming into the limelight[10].

Many studies on the effect of synthetic progestins on mammary gland development and oncogenesis come from rodent experiments. The only species, however, that has comparable or even higher incidences of mammary carcinomas compared to women are female dogs[11]. Early spaying dramatically reduces the mammary cancer incidence in dogs confirming the role of sex steroids in the development of the disease[11, 12]. Canine mammary tumors are characterized by a high homology with human breast cancer with respect to tumor-related signaling pathways and they are often, at least initially, hormone dependent[13]. The majority of malignant mammary carcinomas in the dog are of epithelial origin, but also concurrent tumors of non-epithelial origin may be present and tumors are more heterogeneous as shown by histopathology[14]. This may reflect a higher variation of differentiation of cancer stem cells.

Treatment of beagle bitches with MPA resulted in mammary hyperplasia and neoplasia[15]. A concomitant rise in plasma growth hormone (GH) concentrations suggested that elevated exposure to pituitary GH was

the cause of the mammary tumors in the dog. In 1995, however, we demonstrated that MPA induced local mammary expression of GH[16-18].

Progesterone plays an important role in ductal side-branching and subsequent lactogenic differentiation of the mammary gland. For its effects progesterone uses progesterone receptors (PR) which are present in two forms, a N-terminally truncated form called PR-A and a longer PR-B[19]. Studies using knock out (KO) mice have shown that, at least in mice, PR-B is essential for mammary development. The PRBKO mouse does not develop fully differentiated mammary glands whereas the PRAKO does[20, 21]. These data were the impetus for us to study the PR isoforms in the dog. We found that the dog, as well as the other members of the canidae family (wolf, red fox and raccoon dog), have mutations and insertions in the B-upstream segment (BUS) of PR-B resulting in low to absent transactivation of genes containing progesterone response elements (PRE) in their promoter region. The major mutations causing this are related to the two LxxLL motifs and a tryptophan residue that play a role in the binding of additional co-activators to the PRE complex in the promoter regions of progesterone-regulated genes[22, 23]. Despite the loss of this important signal transduction path dogs still normally develop mammary tissue and, moreover, have a high incidence of mammary neoplasia.

To further study the genes regulated by the canine PR-B we generated a canine mammary cell lines that expresses the cPR-B, human PR-B (hPR-B) or the chimera of canine PR-A and human BUS region (hBUS), under a doxycycline-sensitive promoter[22]. Here we report on the comparison of the expression profile of genes regulated by the progesterone-activated cPR-B, hPR-B and hBUS.

Material and methods

PR-B expressing cell lines and culture

Canine mammary cell line CNMm was kindly provided by Prof. N. Sasaki[24]. This cell line is derived from a metastatic regional lymph node of a canine mammary carcinoma characterized by clinical stage II and a tubular morphology. The cell line does not express progesterone receptors as shown by western blot analysis and the absence of activation of an MMTV-luciferase construct by progesterone (data not shown). This cell line was

stably transfected with a tetracyclin repressor before transfection with the various PR-B construct as described previously[22]. The PR-A translation initiation codons were mutated into alanine to prohibit PR-A expression. Cell lines were cultured in DMEM:F12 supplemented with 10% tetracycline negative FBS (PAA), 3 µg/ml blasticidin (Invitrogen) and 400 µg/ml zeocin (Invitrogen).

Cells were seeded in 6-well plates (Primaria, BD Biosciences, Breda, The Netherlands) at 200,000 cells/well. After 24 h of culture medium was changed to DMEM:F12 supplemented with 5% charcoal-stripped FBS and next incubated for 24h with control medium or in the presence of 1 µg/ml doxycycline, 20 nM progesterone, or the combination of the two. After 24h total RNA was isolated from cells and treated with deoxyribonuclease using RNeasy mini kit (Qiagen, Venlo, The Netherlands).

Gene profiling

RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Science, De Meern, the Netherlands) and RNA integrity was determined using a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, the Netherlands). A two-color DNA microarray with a reference experiment design was performed on 44 k Canine Gene Expression Microarrays V1 (G2519F, Agilent Technologies, Amstelveen, the Netherlands)[25]. Double round of RNA amplification and labeling were performed as described before[26] on an automated system (Caliper Life Sciences, 's-Hertogenbosch, the Netherlands) with 10 to 50 ng total RNA input from each sample. The common RNA reference pool consisted of a mixture of the various RNA preparations. Hybridizations were performed on an HS4800PRO system with Quad Chambers (Tecan Benelux B.V.B.A., Giessen, The Netherlands), using 300 to 500 ng labeled cRNA per channel as described previously[27]. From each incubation, 1 sample was labeled with Cy5 and hybridized against the common reference cRNA (Cy3) on dual channel arrays, and one sample was hybridized in dye swap. Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 100% photomultiplier tube (PMT). After automated data extraction using Imagene 8.0 (BioDiscovery, El Segundo, CA, United States of America), print-tip loess normalization was performed[28] on mean spot intensities. Dye bias was corrected based on a within-set estimate[29]. Data were analyzed using statistical analysis of microarray (SAM)[30] using two class unpaired statistics.

Genes with a *P*-value < 0.05 after Benjamini-Hochberg determination of false discovery rate (FDR) were considered significantly changed; a change cutoff value of 1.5-fold was applied. Differentially expressed genes were converted to their human homologues, and the array comparisons were included in functional pathway analysis using the GeneGo MetaCore platform[31].

Real time quantitative RT-PCR

For qPCR from the isolated RNA cDNA synthesis was performed using iScript kit (BioRad). Specific primer sets were used to amplify gene products (Table I). The reactions were performed and measured using BioRad MyIQ detection system (BioRad) with SYBR green fluorophore. Relative target gene expression was normalized to that of the reference gene Rps19 and relative induction of gene expression was statistically assessed using paired, 2-tailed student's T-test. Relative expression was calculated by the delta-delta Ct (ΔΔCt) method[32].

Four RNA samples (control, cPR-B, hPR-B and hBUS) obtained after treatment with progesterone and doxycycline were also used for miRNA expression profiling. For this purpose, 250 ng total RNA was used for the synthesis of cDNA using the miScript II RT kit (Qiagen). The relative expression was measured using the miScript miRNA Dog miFinder PCR array (MIFD-001Z; Qiagen) on a single 386 well plate using the miScript SYBR Green PCR kit and standard amplification protocol according to the manufacturer. Data analysis was done online (http://pcrdataanalysis.sabiosciences.com/mirna) based on the ΔΔCt method.

Proliferation analysis

Cell proliferation was determined using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, The Netherlands). Cells were seeded in 96 well plates at a density of 6,500 cells/100 µl and cultured in DMEM/F12 medium plus 5% Tet free charcoal stripped FBS. Cells were incubated for 24h with medium alone (control) or in the presence of 1 µg/ml doxycycline and 20 nM progesterone. After 24h 20µl MTT was added in Hanks balanced salt solution and incubated for another 2h at 37°C. Then medium was removed and cells were dissolved using 100 µl DMSO. Finally the absorbance was measured at 595 nm with a reference reading at 650 nm. The background absorbance

intensity was subtracted and cell viability was calculated as a percentage of the absorbance of non-treated cells.

Table I | Primers used for qPCR

Gene	Accession ID	Primer	Sequence (5′ to 3′)
IL-8	NM_001003200	Forward	CTGTTGCTCTCTTGGCAGC
		Reverse	GGGATGGAAAGGTGTGGAG
VEGF	XM_534520	Forward	ATGCGGTGTGGGGGCTGCTGTAATG
		Reverse	TCTCCCCTATGTGCTGGCCTTGATGA
CDKN1	XM_532125	Forward	ATGAAATGGGGGAAGGGTAG
		Reverse	AATCTGTCAGGGGCGTATTG
EGFR	XM_533073	Forward	GGCTCAGGCAAACCACAC
		Reverse	CCGGCAGGGGATGACGAT
FKBP5	XM_538880	Forward	GGAGAAGACCATGACATTCCA
		Reverse	AGCTCAGCATTAGGCTCGAT
SGK	AY514764	Forward	TGGGCCTGAACGACTTTATT
		Reverse	GAGGGGTTGGCATTCATAAG
MT1	NM_001003173	Forward	AGCTGCTGTGCCTGATGTG
		Reverse	TATACAAACGGGAATGTAGAAAAC
RPS19	XM_533657	Forward	CCTTCCTCAAAAAGTCTGGG
		Reverse	GTTCTCATCGTAGGGAGCAAG

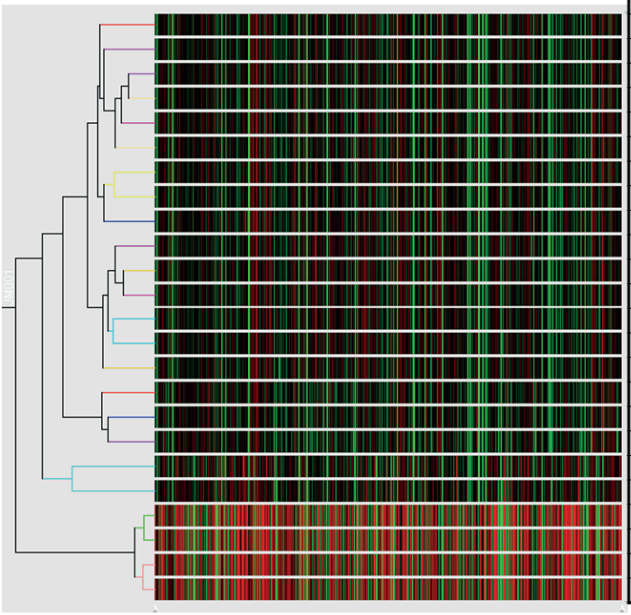
Results

From the cultured cells high quality RNA was isolated with RNA integrity numbers of the samples that varied from 9.2 to 10.0 (mean ± SD; 9.8 ± 0.3), indicating that these samples were excellent for gene expression analysis.

A heatmap of the expression profiles (Fig. 1) clearly showed excellent dye swaps and a close clustering of the differently expressed genes after incubation of the hPR-Band the hBUS cells with the combination of doxycycline and progesterone. The cells expressing the canine PR-B clustered separately from all samples obtained after control incubation or the treatment with doxycycline or progesterone alone. However, the number of differently regulated genes appeared to be much lower. The controls and doxycycline or progesterone treated cells were mutually undistinguishable.

Statistical analysis of the expression data using the SAM analysis tool revealed that the activation of hBUS changed the expression of 693 genes, the hPR-B some 518 genes, whereas the activated cPR-B changed the expression of only 40 genes from which 29 were upregulated and 11 downregulated. These 40 genes (Table II) were all found in the hBUS and hPR-B group indicating that only a subset of genes seemed to be sensitive towards cPR-B. No significant gene expression was found after incubation with doxycycline or progesterone alone.

Figure 1 | Conditional clustering of differently regulated genes at various experimental conditions. Gene profiles were made of canine mammary cell line CNMm stably transfected with doxycycline (dox) inducible PR-B constructs of dog (cPR-B), human (hPR-B) or a chimera of human BUS and canine PR-A (hBUS). Cells were incubated for 24h without additions or after treatment with 1 µg/ml dox, 20 nM progesterone (P4) or a combination of dox and P4. All conditions were tested in duplicate including a dye swap.



Origin	Dox	P4
cPR-B		
hBUS		
hPR-B		
hPR-B		X
hPR-B	X	
hPR-B		X
cPR-B	X	
cPR-B	X	
cPR-B		X
hBUS		
hBUS	X	
hPR-B	X	
hBUS		X
hBUS		X
hBUS	X	
cPR-B		
cPR-B		X
hPR-B		
cPR-B	X	X
cPR-B	X	X
hBUS	X	X
hBUS	X	X
hPR-B	X	X
hPR-B	x	X

Table II | Genes with a fold change >1.5 found after the expression and activation of the cPR-B in comparison to changes in hPRB and hBUS activated cells.

Gene	Name	Gene function	Fold change		
Down			cPRB	hPRB	hBUS
IL8	Interleukin 8	activation of neutrophils	0.40	0.37	0.37
Q3HTT3	Osteopontin	secreted phosphoprotein	0.51	0.52	0.51
ARG2	Arginase	reguation nitric oxide formation	0.53	0.37	0.41
HAPLN1	Hyaluronan and proteoglycan link protein 1	stabilizes proteoglycans	0.54	0.60	0.45
COL14A1	Collagen 14	adhesion	0.60	0.52	0.44
NPTX2	Neuronal pentraxin II	neuronal synaptic protein	0.63	0.46	0.48
TMEFF2	Transmembrane protein	neuronal survival factor	0.63	0.51	0.46
ERG	Transforming protein ERG	ets family of transcription factors	0.63	0.38	0.39
PLK2	Polo-like kinase 2	cell division	0.64	0.53	0.45
GJA1	Gap junction protein alpha	connexin 43 family	0.65	1.01	0.93
Up					
MT2	Metallothionein-2A	binds Zn	2.78	11.45	11.69
HSPB1	Heat shock protein 27	cellular stress	2.31	2.47	2.60
Q32KH1	Sulfatase 2	co-receptor for growth factors/cytokine	2.04	3.16	1.59
DPP4	Dipeptidyl-peptidase 4	T-cell activation	1.94	1.36	1.11
OSBPL3	Oxysterol binding protein-like 3	intracellular lipid receptor	1.94	4.26	6.43
MT1	Metallothionein-1	binds heavy metals	1.93	8.53	8.87
ABLIM1	Actin binding LIM protein 1	cytoskeleton	1.85	2.38	2.58
ZP2	Zonapellucida glycoprotein 2	acrosome reaction	1.82	20.55	21.93
ANGPTL2	Angiopoietin-like 2	angiogenesis	1.81	2.39	2.36
PLAT	Plasminogen activator, tissue	plasminogen activator	1.74	3.37	3.69
PDLIM2	PDZ and LIM domain 2	promotes cell migration and adhesion	1.73	1.51	1.03
FLNC	Filamin C, gamma	cytoskeleton	1.70	.64	0.63
FILIP1L	Filamin A interacting protein 1-like	regulator of anti-angiogenic activity	1.68	3.13	4.22
SOX9	SRY (sex determining region Y)-box 9	chondrocyte differentiation	1.65	1.04	0.98
ADAM9	ADAM metallopeptidase domain 9	metalloproteinase	1.63	1.78	1.93
CTGF	Connective tissue growth factor	Mitogen	1.62	4.60	5.34
LOXL3	Lysyl oxidase-like protein 3	biogenesis of connective tissue	1.57	1.57	1.70
PMP22	Peripheral myelin protein 22	growth regulation	1.57	2.93	3.70
CCBL1	Cysteine conjugate-beta lyase	transamination	1.55	4.29	4.94
TSC22D3	TSC22 domain family, member 3	transcriptional regulators	1.54	1.68	1.55
LAP3	Leucineaminopeptidase 3	processing of intracellular proteins	1.54	3.03	3.12
PLOD2	Lysyl hydroxylase 2	intermolecular collagen cross-links	1.54	1.79	2.01
AGRN	Agrin proteoglycan	neuromuscular junction	1.53	1.01	1.77
SGK	Serum/glucocorticoid regulatory kinase	cellular stress	1.51	1.01	0.91
BCKDHB	Branched chain keto acid dehydrogenase E1	catabolism of branched chain AA	1.50	2.49	2.55

Table III | Enrichment analysis of the genes influenced by progesterone in the canine mammary cell line CNMm expressing hPR-B or hBUS.

#	Network	Genes
1	Regulation of angiogenesis	HB-EGF, c-Rel (NF-kB subunit), PGAR, Galpha(q)-specific peptide GPCRs, PDK (PDPK1), CXCR4, I-kB, Galpha(i)-specific peptide GPCRs, PLAUR (uPAR), IP3 receptor, CTGF, VEGF-A, IL-8, G-protein alpha-q/11, IGFBP7/8
2	Blood vessel morphogenesis	HB-EGF, c-Rel (NF-kB subunit), DEP-1, PGAR, Galpha(q)-specific peptide GPCRs, PDK (PDPK1), PLAT (TPA), CXCR4, Galpha(i)-specific peptide GPCRs, CTGF, VEGF-A, G-protein alpha-q/11, IGFBP7/8
3	G1-S Interleukin regulation	c-Rel (NF-kB subunit), NFKBIA, PDK (PDPK1), Tob1, I-kB, IL-8, Cyclin D1
4	NOTCH signaling	SFRP3 (FRZB1), FZD2, HB-EGF, c-Rel (NF-kB subunit), FZD6, NFKBIA, PDK (PDPK1), I-kB, Frizzled, VEGF-A, Cyclin D1
5	ERBB-family signaling	GAB1, HB-EGF, PDK (PDPK1), EPS8, I-kB, MKP-1
6	WNT signaling	FZD2, HB-EGF, Frizzled, PLAUR (uPAR), IP3 receptor, VEGF-A, G-proteinalpha-q/11, G-proteinalpha-q, Cyclin D1

Table IV | Analysis of genes commonly regulated by cPR-B, hPR-B and hBUS

#	Network	Genes in common
1	Development: Regulation of angiogenesis	IL-8, CTGF, PGAR, Galpha(q)-specific peptide GPCRs, Galpha(i)-specific peptide GPCRs, IGFBP7/8, CXCR4
2	Development: Blood vessel morphogenesis	PLAT (TPA), CTGF, PGAR, Galpha(q)-specific peptide GPCRs, Galpha(i)-specific peptide GPCRs, IGFBP7/8, CXCR4
3	Reproduction: Feeding and Neurohormone signaling	HSP27, ADAM9, Galpha(q)-specific peptide GPCRs, Galpha(i)-specific peptide GPCRs,
4	Cell adhesion: Platelet-endothelium-leucocyte interactions	PLAT (TPA), IL-8, CTGF, IGFBP7/8
5	Proteolysis: ECM remodeling	Collagen XIV, PLAT (TPA), CTGF
6	Cell adhesion: Leucocyte chemotaxis	IL-8, Galpha(q)-specific peptide GPCRs, Galpha(i)-specific peptide GPCRs, CXCR4
7	Chemotaxis	IL-8, Galpha(q)-specific peptide GPCRs, Galpha(i)-specific peptide GPCRs

Table V | Selection of miRNAs showing a higher than two fold (bold) difference after expression and activation by progesterone of hPR-B, cPR-B or hBUS in canine mammary cells.

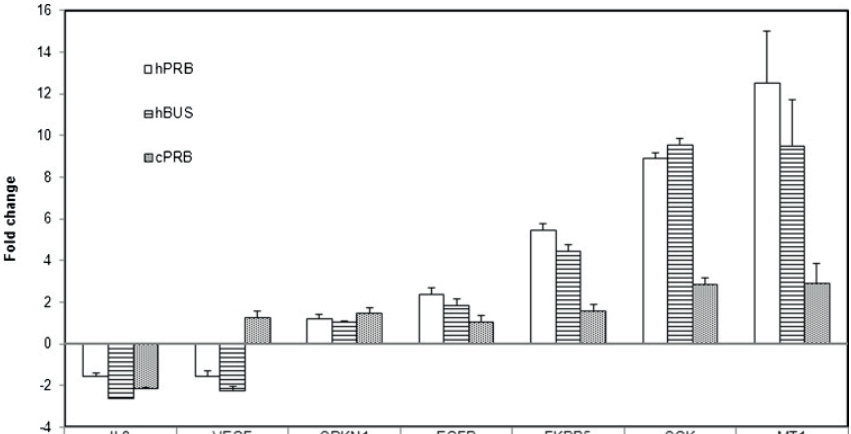
miRNA	cPRB	hPRB	hBUS	Threshold	Target
Let 7a	-1.47	**-2.40**	-1.27	OK	Ras, HMGA2
miR-101	1.15	1.30	**2.02**	>30	-
miR-124	-1.94	**-2.33**	-1.25	>30	SCP1
miR-192	**-2.31**	-1.14	1.25	>30	p53
miR-205	**-3.47**	**-2.27**	-1.68	>30	E-Cadh, ZEB1, ZEB2
miR-210	-1.14	1.87	**3.52**	OK	-
miR-214	-2.02	-1.37	**2.12**	OK	MAP2K3, MAPK8
miR-29b	-1.92	**2.08**	**2.08**	>30	MCL1, TCL1A
miR-375	**-2.45**	**-2.40**	1.47	>30	MTPN, PDK1

Enrichment analysis by GeneGo showed that the hBUS and hPR-B had 408 genes in common. The most prominent networks influenced by progesterone treatment included (1) development/ angiogenesis, (2) cell adhesion/ECM remodeling, (3) signal transduction/ Wnt, Notch and ERBB-family (Table III). For the genes that the activated cPR-B expressing cells had in common with the hBUS and hPR-B regulated genes a role in cell adhesion, EMT and angiogenesis was found predominantly by the expression of IL-8, CTGF, PLAT, IGFBP7/8, CXCR4 and GPCRs (Table IV).

From a selection of genes the expression was analyzed by qRT-PCR (Fig. 2). A close correlation between hPR-B and hBUS regulated genes was found (R^2=0.96). From the cPR-B tested genes IL-8 expression was clearly inhibited, no change was seen in the expression of VEGF, CDKN1 and EGFR whereas FKBP5, SGK and MT1 were upregulated, however to a much lower extent in comparison to hPR-B and hBUS.

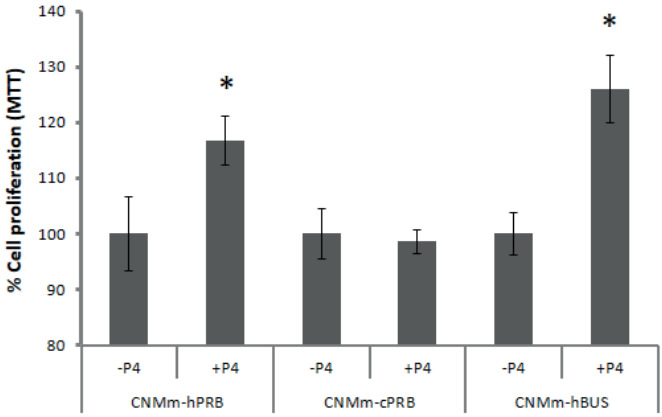
After incubation for 24h with 20 nM progesterone in the presence of doxycycline increased cell proliferation was found in the cell lines containing hPR-B and hBUS whereas no effect at all was found in the cPR-B expressing cells (Fig. 3).

Figure 2 | qPCR results of selected target genes. Given are the fold changes after incubation with doxycycline and progesterone in comparison to untreated controls.



	IL8	VEGF	CDKN1	EGFR	FKBP5	SGK	MT1
hPRB	-1.59	-1.56	1.20	2.36	5.44	8.88	12.5
hBUS	-2.63	-2.22	1.03	1.87	4.46	9.57	9.52
cPRB	-2.12	1.26	1.47	1.06	1.61	2.84	2.9

Figure 3 | Cell proliferation of canine mammary carcinoma cell line CNMm before and after treatment with progesterone (P4) in the presence of doxycycline.



Finally, a preliminary screen of changes in the expression of miRNAs was applied using a dog-specific miRNA PCR array (Table V). Of the 84 tested miRNAs only 9 gave a fold change higher than 2 in either one or more samples tested in comparison to control. No clear relation was found between the effects of activated hPR-B and hBUS, whereas in the cPR-B sample some changes were noticed. Of the putative genes regulated by these miRNAs only MAP2K3, MCL1 and PDK1 also showed to be differently regulated in the microarray data.

Discussion

In a previous study we reported on the fact that the canine PR-B showed no transactivation of a PRE2-luciferase reporter construct, specific for PR-B, whereas a low transactivation potential was found on a MMTV-luciferase construct that reflects PR activation more in general[23]. In this paper we extended this study to the expression profiling of endogenous genes in a canine mammary cell lines expressing doxycycline-inducible hPR-B, cPR-B or hBUS.

In comparison to non-treated controls the addition of doxycycline alone did not result in any change of the expression profiles. This brings us to the conclusion that obviously the doxycycline concentration used was not toxic and that expression of the PR-B in itself has no effect on the expression of other genes. This was true for all three receptor variants. Our findings contrast with the reported ligand-independent effects of the PR in breast cancer cells[33] or brain tissue[34]. This may, however, be due to the fact that those ligand-independent effects are in general fast[35] and may need activation by EGF[36] or neurotransmitters[34], and cannot be found in our experimental setup.

Also treatment with progesterone alone did not change expression profiles indicating that in the absence of PR-B these cells did not respond via binding of progesterone to other proteins such as the progesterone membrane receptors[37-40]. This was not due to the absence of these receptors as both PGRMC1 and PGRMC2 were highly expressed according to the microarray (data not shown). Only the combination of doxycycline-induced PR-B expression in combination with activation

by progesterone resulted in the expected changes in gene expression profiles in case of the hPR-B and hBUS. Pathway analysis of the differently regulated genes showed changes in pathways that are in general affected when human and canine mammary carcinomas are compared[41, 42]. The activated cPR-B, however, hardly changed the expression profile confirming the earlier reported lack of transactivation potential due to changes in the activation function 3 (AF3) domain in the BUS region[23]. This raises the question whether the cPR-B has a very limited transactivation potential or that the dog and other members of the canidae family are in essence natural functional PR-B knockouts. The fact that the progesterone-activated cPR-B did not transiently stimulate the proliferation of the CNMm cells whereas both hPR-B and hBUS did further demonstrate the absence of a biological function.

The latter challenges the question on what the function of PR-B is in mammary development and the pathogenesis of breast cancer. Studies using knockout mice showed that not the PR-A isoform, but rather PR-B is essential for mammary development[43, 44]. If dogs and other canids are functional PR-B knockouts but they can develop normal mammary glands, this may be true also for other species. No studies are presented to our knowledge with respect to the role of PR-isoforms in human mammary development, but in humans the overexpression of PR-A, and/or the loss of PR-B, is associated with a more malignant mammary phenotype[45, 46]. The question is whether this explains the high incidence of mammary tumors in dogs having only a functional PR-A, and if PR-A alone is sufficient in humans to stimulate mammary development and carcinogenesis.

Alternatively, dogs may still have a limited but important residual PR-B activity. A few genes showed differential expression in our experiment. Among them is IL-8 showing decreased expression in the microarray, confirmed by RT-PCR. Decreased IL-8 expression has also been found in human and canine malignant mammary carcinomas but no relation to PR was investigated[47, 48]. Progesterone may also play a role in angiogenesis. Again IL-8 may be involved but in contrast with hPR-B and hBUS no effect was shown on the expression of VEGF. The progesterone-activated cPR-B stimulated the expression of FKBP5, SGK, and MT1 although to a much lower extent than hPR-B and hBUS did. With the exception of FKBP5 all

other genes are regulated in a modified T47D human breast cancer cells also when only the PR-A isoform is expressed[49] in line with the idea that there may be some crosstalk of the overexpressed cPR-B with PR-A regulated gene expression.

The primary screen of miRNA expression resulted in a few differences that need further exploration. Of the 9 differently expressed miRNAs 6 had Ct values above 30 cycles what limits the interpretation. Studies on progestin regulation of miRNA expression in human T47D cells, which express both PR-A and PR-B, also showed that the effects were transient and not significant after 24h of treatment[50]. The only point to be made here is that the cPR-B did influence the expression of some miRNAs suggesting a biological role for cPR-B.

Finally, a remark should be made on the test system that we used that may be artificial. First, the doxycycline-induced expression resulted in supra-physiological mRNA and protein expressions that may have caused the limited cPR-B regulated changes in gene expression but may be not physiologically significant. From our previous study using PRE-containing reporter constructs we know also that these constructs could be activated by cPR-B to a very limited extent although considerably lower than hPR-B and hBUS[23]. The major changes found in the AF3 domain or BUS area of cPR-B certainly limit the nuclear transactivation potential. In the human PR-B thus far only the effects of mutations are studied in the areas responsible for interaction with the estrogen receptor (ERα), DNA binding site or dimerization area[51] but not in the PR-B specific BUS area. The canine cell line used in this study does also not express the ERα gene that plays an important role in cytoplasmic signaling of PR-B and therefore a role in cytoplasmic signaling cannot be ruled out. However, specific cytoplasmic effects of hPR-B have been described in cells lacking ERα[52].

In conclusion we showed a very limited transactivation potential of the canine PR-B on endogenous genes using microarray analysis. We cannot conclude with 100% certainty that the dog is a natural and spontaneous functional PR-B knockout. And thus the question remains if the PR-B is necessary for mammary gland development and carcinogenesis, at least in the dog but maybe also in humans.

Acknowledgements

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CHAPTER 4

Expression of Progesterone Receptor B in Canine Mammary Tissue

A. Gracanin, M. Bominaar and J.A. Mol

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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Abstract

Progesterone receptor gene encodes a shorter PR-A isoform and a longer PR-B isoform that contains the PR-B specific activation function (AF-3) domain. Canids have lost AF3 domain dependent transcriptional activity of progesterone receptor due to numerous mutations in essential sequence motifs that are otherwise highly conserved in mammals. In this study we tested the hypothesis that dogs, and likewise other canids, were able to accumulate these mutations because they have lost PR-B expression and are as such a natural PR-B knock out. Attempts to detect PR-B in canine mammary cell lines and tissue by western blot using an antibody recognizing both progesterone receptor isoforms were either unsuccessful or resulted in detection of putative PR-B protein bands with molecular weights slightly higher in comparison to protein expressed from canine PR-B construct. To further analyze PR-B expression we generated PR-B specific antibodies. The KAQD antibody showed the highest specificity for canine PR-B and only a weak cross-reactivity with other canid and non-canid species. Western blot results using KAQD antibody on canine mammary cell lines and tissue extracts were inconclusive regarding the previously detected putative PR-B band. Interestingly, progestin exposed canine mammary tissue showed a potent induction of RANKL expression that in mice is uniquely PR-B regulated. In conclusion, the data implicates the absence or very low expression of PR-B in canine mammary tissue, but still a conservation of downstream progesterone signaling in the mammary gland involving induction of RANKL expression.

Introduction

The hormone progesterone regulates essential aspects of female reproductive biology and has been identified as an important risk factor for development of mammary tumors in dogs and in humans[1-3]. Progesterone signals through progesterone receptor (PR), a member of nuclear receptor family. PR is expressed in two isoforms, PR-B and PR-A, originating from one human gene through usage of two distinct promoters[4]. PR-B isoform has an additional N-terminal segment containing activation function 3 (AF3) domain that is essential for its transactivation potential on target genes[5,6]. We have recently reported that the N-terminal region of canine PR-B has an unusually high GC content and that canid PR-B (including canine PR-B), uniquely among mammals, lacks AF3 domain-dependent activity[7]. Accumulation of multiple mutations in canid AF3 domain implicates a loss of selective pressure to conserve the regarding sequence. In this study we address the proximate cause for the loss of AF3-domain function in dogs and other canids. One explanation could be that in the common ancestor of all canids, the first mutation in AF3 domain resulted in a major loss of function, thereby removing the selection pressure to conserve the sequence. According to this hypothesis all canids should carry a common mutation in one essential AF3 domain residue[6] that would be sufficient to dramatically decrease its function. Based on the previously reported sequence alignment of all available mammals (including canids) PR-B specific region[7], in the second AF3 domain LxxLL motif there is a canid specific mutation of the second leucine residue into alanine. Although simultaneous mutation of all three leucines within LxxLL motif was shown to be deleterious to the function[6], mutation of a single leucine residue within the motif is present in at least 6 other mammalian species implying it may not be as deleterious. An alternative scenario is that canids have lost selectively PR-B expression and therefore seem to be a natural PR-B knock out. Loss of PR-B expression would by itself release the selective pressure to conserve the PR-B specific coding and regulatory sequence and enable accumulation of *ad random* mutations. Expression of canine PR has so far mainly been addressed using biochemical (ligand binding) assays[8,9], immunocytochemistry[10] and immunohistochemistry[65], thereby not discriminating between the two protein isoforms. PR-B expression and function is essential for mammary gland development

in mice[12]. Moreover, expression of PR-B was readily detected in human and mouse mammary cell lines and tissues[13,14]. In this study we focused, therefore, on detection of PR-B in canine mammary cell lines and tissues.

Material and methods

Cell lines and tissue

Canine mammary cell lines used in this study were established from primary tumors or its metastasis and were described previously[15-17]. Generation of CNMm cells stably expressing inducible human or canine PR-B was described previously[7], as was CHO-K1 cell line[18]. The cells were cultured on DMEM:F12 (Invitrogen, The Netherlands) supplemented with 10 % FBS (FBS Gold, PAA). Canine mammary tissue from progestin (MPA) treated dogs and from healthy control dogs and detailed animal experiment protocols have been previously described[19].

Transfections and luciferase reporter

For protein expression analysis, CHO-K1 cells were seeded in 6-well plates (Primaria) at 200,000 cells/well, 48h before transfection. Transfections were performed with 10 µl Lipofectamine 2000 (Invitrogen) and 1 µg PR constructs[7] per well. CNMm-hPR-B or -cPR-B cells were seeded in 6-well plates (Primaria) at 400,000 cells/well 24h prior to 24h treatment with 1 µg/ml DOX (Sigma). For luciferase reporter assay, cells were seeded in 24-well plates (Primaria) at 70000 cells/well, 48h before transfection. Transfections were performed in absence of FBS using, per well, 2 µl Lipofectamine 2000, 800 ng MMTV-luc[18], 0.3 ng human β-actin-promoter renilla[20] as an internal control and 10 ng PR constructs[7] or empty pCl-neo vector. After 5h the medium was changed to DMEM:F12 or MEM supplemented with 5 % charcoal-stripped FBS (PAA). Cells were left to recover for 24h, followed by a 24h treatment with 20 nM progesterone. The firefly and renilla luciferase activities were subsequently quantified using Dual luciferase assay system (Promega) in a Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium).

Immunohistochemistry and western blotting

For immunohistochemistry, formalin-fixed paraffin-embedded mammary tissue samples were used. Slides of 10 µM were deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. Peroxidase-

blocking was performed using 3% H_2O_2 in TBS for 15 min. Antigen retrieval was carried out by boiling in 50 mM citrate buffer for 20 min and then left to cool down for another 20 min. Nonspecific antibody binding was blocked using 10% normal goat serum and 1% BSA for 30 min at RT. Slides were next incubated using a PR antibody (SC-539; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1500 and kept overnight at 4°C. The secondary antibody (Envision anti-rabbit, K4003; DAKO, Heverlee, Belgium) was incubated for 30 min and then washed off. The slides were then incubated with chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) to visualize the immunoreactions. Finally, they were covered with haematoxylin (Hematoxylin QS, Vector H3404; DAKO, Heverlee, Belgium) for 5s, dehydrated and then mounted with Vecta mount (Vector H-5000). For expression analysis in mammary tissue, protein was isolated from tissue dissolved in 0.5 ml lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), containing 1.5 mM EDTA, 10 mM sodium molybdate, 10% glycerol, 1 mM DTT, 1 M sodium orthovanadate, 1 mM PMSF and 10 µg/ml aprotinin. Tissues were homogenized on ice using an Ultra Turrax tissue grinder. Next, samples were centrifuged for 20 min at 16 000 g and 4°C. In the supernatant, protein concentration was determined using BioRad Dc Protein Assay (BioRad). Fifty microgram protein of total cell lysates was subjected to SDS-PAGE and analyzed by Western blot using primary antibody against human PR (sc-539 or Ab8 (Thermo Scientific)) and the secondary goat anti-mouse or goat anti-rabbit HRP-conjugated antibody (HAF007 and HAF008, respectively; R&D Systems, UK). HRP was visualized using Advance TM-Enhanced chemiluminescence (ECL, Amersham; GE Healthcare, Eindhoven, The Netherlands) and analyzed using GelDoc2000 (BioRad). For expression analysis in cell lines, protein isolation and western blotting was performed as described previously[7]. Canine PR-B specific polyclonal antibody production (including peptide synthesis, immunization and subsequent affinity purification) was performed by DavidsBiotechnologie GmbH (Regensburg, Germany). After successful immunization with three synthetic fragments of the canine PR-B, antibodies were isolated against these three fragments starting with the sequences TERT, AAEP and KAQD. Affinity purified TERT, AAEP and KAQD antibodies were used at optimal 1:1000 dilution.

Quantitative real-time PCR

Total RNA from cell lines and tissue was isolated and treated with DNase using RNeasy mini kit (Qiagen, The Netherlands) according to manufacturer's

protocol. cDNA synthesis was performed using iScript kit (BioRad). Specific primer sets used to amplify gene products are depicted in the table below. The reactions were performed and measured using BioRad MyIQ detection system (BioRad) with SYBR green fluorophore. Relative target gene expression was normalized to that of the reference gene Rps19 and relative induction of gene expression was statistically tested using paired, 2-tailed student's T-test.

Gene	Forward primer	Reverse primer
Rps19	5'-CCTTCCTCAAAAAGTCTGGG-3'	5'-GTTCTCATCGTAGGGAGCAAG-3'
PGR	5'-CAATGGAAGGGCAGCATAAC-3'	5'-CAGCACTTTCTAAGGCGACA-3'
RANKL	5'-AGAGCATTAAGCAGGC-3'	5'-TATGAGTCTTGCCCCT-3'

Results

Assessment of PR expression in canine mammary cell lines and tissue

Multiple attempts to identify the 5'-UTR regions of canine PR isoforms failed and were attributed to a very high GC content of the canine sequence ([21] and data not shown). Based on the human sequence it is expected that the 5'-UTR of PR-A overlaps with the PR-B specific coding region[4], disabling us from designing primers specific for each PR isoform. Alternatively, a panel of canine mammary cell lines was screened for PR expression using primers amplifying both isoforms. PR mRNA was detectable in all cell lines, but the expression levels were variable (Fig.1A). However, protein levels of PR, assessed by western blotting, were undetectable (data not shown). To alternatively test for PR protein expression, cell lines were transiently transfected with progesterone responsive MMTV-luciferase reporter. None of the cell lines showed MMTV-luciferase reporter activity upon treatment with progesterone, but all of them responded potently upon co-transfection with either human or canine PR-B (Fig.1B). Lack of PR expression in canine mammary cell lines, prompted us to analyze canine mammary tissue. Immunohistochemical analysis using general PR antibody sc-539, showed positive nuclear and weak cytoplasmic staining in epithelial cells in proliferative zones of the mammary gland, whereas in differentiated mammary tissue, the number of PR-positive cells was low (Fig. 1C and [21]). Western blot analysis of canine mammary tissue with sc-539 antibody

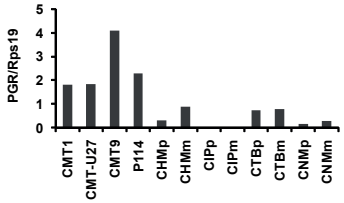
revealed prominent staining at molecular weights corresponding to PR-A (82-94 kDa[7]) (Fig. 1D). Using the same antibody, we have previously shown that the cPR-B is expressed as a 108 kDa protein when transfected in canine mammary cell lines compared to the hPR-B of 116 kDa[7]. A putative PR-B band was also detectable in canine mammary tissue, but the molecular weight appeared higher than 108 kDa (Fig. 1D).

Development and assessment of canine PR-B specific antibodies

PR-B specific N-terminal region of the protein is known to have a low level of conservation. Conservation appears to be restricted to functionally important residues such as AF3 domain motifs[22]. Consequently, in contrast to antibodies that recognize the common PR-A sequences, currently

Figure 1 | (A) Average relative mRNA expression of PR in canine mammary cell lines. **(B)** Average MMTV-luc transactivation in canine mammary cell lines upon 20 nM progesterone treatment either in basal state or after transfection with PR-B constructs (hPR-B =human PR-B, cPR-B = canine PR-B). **(C)** Immunohistochemical staining for PR in canine mammary tissue. **(D)** Western blot using sc-539 antibody in canine mammary tissue. M= molecular weight marker; N= Normal tissue; H= hyperplastic tissue (after treatment with progestins); C= carcinoma. Putative PR-B and PR-A bands are indicated by arrows.

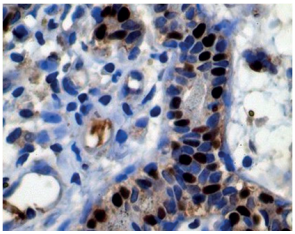
A



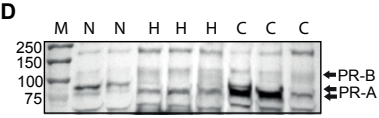
B

Cell line	basal	hPR-B	cPR-B
CMT1	1,03	9,28	
CMT-U27	0,91	52,64	
CMT9	0,95	41,13	
P114	1,14		4,59
CHMp	0,75	43,50	4,26
CHMm	1,33	84,30	
CIPp	1,16	30,30	
CIPm	1,03	23,81	
CTBm	1,88	25,75	
CNMp	1,09	45,53	6,48
CNMm	1,04	25,76	

C



D



available PR-B specific antibodies cannot be used to detect cPR-B. For this reason we attempted to develop and assess new cPR-B specific antibodies. For this purpose one rabbit was immunized with a mixture of three peptides (Fig. 2A) and generated antibodies designated TERT, AAEP and KAQD, were subsequently affinity purified. To test immunoreactivity of the raised antibodies against canine PR-B, western blot was performed on protein extracts from empty or canine PR-B-transfected CHO-K1 cells and compared to sc-539 antibody and to non-affinity purified serum. Immunoreactivity with canine PR-B protein was observed with AAEP and KAQD antibodies with latter showing less non-specific binding (Fig. 2B). TERT antibody had a low titer (data not shown), showed high non-specific binding and had no immunoreactivity to cPR-B (Fig. 2B) and was therefore discarded from further analysis. We next tested whether KAQD and AAEP antibodies cross-react with other canid and non-canid species. For this purpose western blot was performed on protein extracts from CHO-K1 cells transiently transfected to express chimeric PR-B proteins consisting of canine PR-A and PR-B specific regions of human, dog, raccoon dog, brown bear and gray seal (Fig. 2C). KAQD antibody showed a strong immunoreactivity against canine PR-B and a weak cross-reactivity with raccoon dog, as well as bear, seal and human PR-B. AAEP showed comparable immunoreactivity between canine and raccoon dog protein, but the additional higher non-specific protein band prevented assessment of cross-reactivity with non-canid proteins. Next, KAQD antibody was used to assess canine PR-B expression in cell lines and tissues reported or expected to express the protein. Recently, expression of two PR isoforms was reported in two clonal cell lines derived from CHMp cells, namely CHMp-5b and CHMp-13a[23]. To confirm these findings we performed western blot on CHMp-5b and CHMp-13a cell lines and CNMm cells stably expressing inducible canine or human PR-B[7]. Surprisingly, Ab-8 antibody used previously to assess PR expression in CHMp-5b and CHMp-13a cells, reacted strongly with human PR-B, but it did not recognize the induced cPR-B protein in CNMm cells. Similarly, no PR-B like protein band was detectable in the two clonal cell lines. Staining with sc-539 and KAQD antibodies confirmed these findings (Fig. 2D). Lastly, KAQD antibody was used on canine mammary tissue protein extracts previously analyzed with sc-539. Similarly to sc-539, multiple protein bands were detected. KAQD did recognize a broad protein band (smear) of approximately 100-110 kDa, but this did not fully overlap with the putative PR-B band recognized by sc-539 (Fig. 2E). Based on these data, we are unable to provide evidence for

expression of canine PR-B. This is intriguing, as knock out studies in mice have implicated that progesterone-regulated mammary development is highly dependent on PR-B[12]. Recently, RANKL has been identified as a major downstream mediator of progesterone signaling in the mammary gland[24]. We therefore assessed RANKL expression in the canine mammary tissue before and after exposure to progestins. Interestingly, also in dogs, progestins are capable of potently inducing RANKL expression in the mammary gland (Fig. 2F).

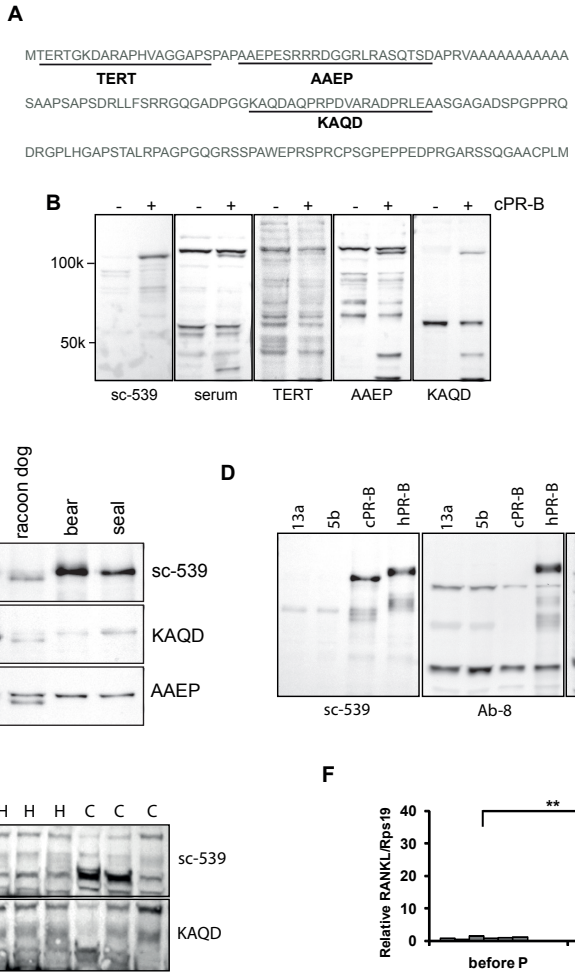
Discussion

Accumulation of multiple mutations and a high GC content of the N-terminal, PR-B specific coding sequence in dogs and other canids, suggests a loss of selection pressure to conserve the sequence. In this study we hypothesized that this is the consequence of a selective loss of PR-B expression in canids.

A sensitive method to detect PR-B expression would be to perform quantitative real-time PCR using primers specific to PR-B isoform. In two recent studies detection and quantification of canine progesterone receptor isoforms was based on PR-B primers designed to amplify PR-B specific coding region[25,26]. Taking into account that 5'UTR region of human PR-A overlaps the PR-B coding sequence[4], PR-B specific primers need to be designed in the 5'UTR specific to PR-B transcript. This information is however lacking for the canine sequence, thereby preventing assessment of PR-B expression at mRNA level. Assessment of total PR expression in canine mammary cell lines suggested variable mRNA expression, but no protein or its activity could be detected in the same cell lines. This may implicate a loss of PR expression at the (post)translational level or amplification of non-specific template. Sequencing of amplified PCR product confirmed the specificity of the sequence (data not shown), but it is interesting to note that additional antisense transcripts overlapping human PR promoter and coding sequence have been detected[27]. As we have not performed a strand specific cDNA synthesis, potential antisense transcripts may also have been amplified during the PCR reaction.

Assessment of endogenous PR-B protein expression in canine mammary tissue using an antibody recognizing both PR isoforms (sc-539) resulted

Figure 2 | (A) Canine PR-B specific N-terminal fragment with marked sequences used for peptide synthesis. **(B)** Western blot of CHO-K1 transiently transfected with canine PR-B (cPR-B) with three affinity purified antibodies. Serum = serum before affinity purification. **(C)** Western blot with sc-539, KAQD and AAEP antibodies on a lysate of CHO-K1 cells transiently transfected with different chimeras. **(D)** Testing CHMp-13a and -5b using sc-539, Ab-8 and KAQD antibodies. **(E)** Comparing sc-539 and KAQD western blots of canine mammary tissue. **(F)** Rps19 normalized RANKL mRNA expression in canine mammary tissue before and after progestin (P) exposure, ** = p<0.01.



in detection of putative PR-B protein band with molecular weight higher than expected based on the molecular weight of the protein expressed from a canine PR-B encoding construct. We additionally generated canine PR-B specific antibodies and tested them for their immunoreactivity with canid and non-canid species. KAQD antibody was the most specific among the generated antibodies, recognizing canine protein and showing weak cross-reactivity with other canid and non-canid species. Western blot using KAQD antibody on canine mammary tissue extracts detected protein bands that do not fully match putative PR-B bands detected by sc-539 antibody, implicating lack of PR-B expression in the analyzed canine mammary tissue extracts. It is however possible that canine PR-B is expressed but that the expression levels are very low and/or have restricted localization within the tissue. Immunohistochemical analysis using PR-B specific antibody could in this case prove more informative. KAQD antibody still needs to be tested for its specificity in immunohistochemical analysis and canine mammary cell line CNMm expressing doxycycline-inducible canine PR-B[7] could serve as a good test system in this regard.

In addition, we question the previously reported data showing that canine mammary cell lines CHMp-13a and CHMp-5b express both PR isoforms[23]. Antibody used in the regarding study (Ab-8) appears not to cross-react with canine PR-B, and sc-539 and KAQD antibodies failed to detect either of the isoforms in the extracts of these cell lines.

Potent induction of RANKL in progestin treated canine mammary tissue is intriguing considering the undetectable expression of canine PR-B. Recently, progesterone induced RANKL expression in mouse mammary cells was shown to be dependent on tethering of Stat5a to PR[28]. Stat5a is known to be activated by multiple growth factors including prolactin and growth hormone (GH)[29]. Interestingly, unlike human or mice, progestin treated canine mammary tissue shows also a more potent induction of growth hormone mRNA expression[30]. It is therefore interesting to hypothesize that induction of growth hormone in the canine mammary gland serves as a compensatory mechanism to induce RANKL expression in the absence or low expression of PR-B.

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CHAPTER 5

Lack of direct crossregulation between growth hormone signaling and canonical Wnt signaling in canine mammary tumor cell lines

Ana Gracanin, Jeroen Vis, Elpetra Timmermans-Sprang and Jan A. Mol

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

Oncogenic potential of progesterone in the canine mammary gland is correlated with induction of mammary growth hormone (GH) expression and activation of canonical Wnt signaling. In this study we tested a model in which progesterone-independent activity of GH and canonical Wnt signaling in canine mammary tumors involves a direct crossregulation between the two pathways. For this purpose, a panel of canine mammary tumor cell lines was screened for their activity and responsiveness of GH signaling. None of the tested cell lines expressed GH nor was responsive to exogenous GH due to insufficient expression of functional GH receptor (GHR) protein. We therefore generated a canine mammary tumor cell line with doxycylcine (DOX)-inducible canine GHR expression (CNMm-cGHR) that upon DOX treatment becomes potently responsive to GH stimulation. Using this cell line we tested whether GH signaling can influence canonical Wnt signaling by measuring TCF reporter activity or Axin2 expression upon stimulation of GHR expression. Neither of the two parameters changed in the regarding cell line. Alternatively, we tested whether activation of canonical Wnt signaling using GSK3β inhibitor CHIR can affect GH or GHR expression in canine mammary cell lines, thereby stimulating the GH signaling. The treatment had, however, no effect on the expression of GH or GHR. Based on this data we suggest that GH and canonical Wnt signaling have no direct crossregulation in the mammary gland and are regulated by progesterone, therefore, putatively independently of each other.

Introduction

The hormone progesterone is recognized as a risk factor for breast cancer[1], but the underlying signaling mechanisms are not fully understood. Non-spayed female dogs show a very high incidence of spontaneous mammary tumors and interestingly, prolonged exposure to progestins alone is sufficient to induce mammary hyperplasia and benign tumors[2,3]. Although majority of mammary tumors express progesterone receptor (PR), in a subset of malignant tumors expression is either downregulated or lost, implying a loss of progesterone control[4,5].

In the normal mammary gland progesterone exerts its action through induction of paracrine factors in PR positive cells that stimulate proliferation of the neighboring PR negative cells[6]. As such, in mice, progesterone has been shown to induce canonical Wnt signaling in the normal mammary gland putatively through induction of Wnt ligands (especially Wnt4)[7,8]. We have recently shown similar progestin-induced activation of Wnt ligands and canonical Wnt signaling in canine mammary tissue[9]. Activation of canonical Wnt signaling in presence of Wnt ligands is characterized by stabilization of cytoplasmic β-catenin and its subsequent translocation to the nucleus where upon binding to TCF/LEF1 family of transcription factors it activates gene transcription[10]. Human breast cancers often show overstabilization of β-catenin implying an active canonical Wnt signaling[11]. One of the mechanisms proposed for this aberrant activation of the canonical Wnt signaling is its crossregulation by other pathways frequently deregulated in tumors (e.g. EGF, PTEN and p53)[12].

Similar phenomenon is observed regarding mammary growth hormone (GH). Progestin-induced hyperplastic and benign canine mammary lesions show overexpression of GH, exclusively in PR positive cells[13,14]. Moreover, increased expression of mammary GH is also observed in human mammary tumors where it is thought to contribute to proliferation and oncogenic transformation of mammary cells in an autocrine and/or paracrine fashion[15-17]. Interestingly, mammary GH expression has been detected also in tumors that have lost PR expression[18] and in normal human breast epithelium the majority of GH positive cells were adjacent to PR positive cells[19]. This suggests involvement of additional (progestin-responsive) signaling pathways in the regulation of mammary GH signaling.

In this study we explore the possibility that progestin-induced canonical Wnt signaling and mammary GH signaling involve direct crossregulation between the two pathways. The existence of such crossregulation mechanism could explain pertained activity of canonical Wnt signaling and GH signaling in mammary tumors that lack PR expression and could putatively be used as a therapeutic target.

Materials and methods

Cell lines

Canine mammary cell lines used in this study were described previously[20-22] as was the generation of CNMm-6TR cell line[23]. The cells were cultured on DMEM:F12 (Invitrogen, The Netherlands) supplemented with 10 % FBS (FBS Gold, PAA). For generation of CNMm-6TR cell line with doxycycline (DOX)-inducible canine GHR (CNMm-cGHR), full length coding region of cGHR was amplified from a cDNA pool of various canine mammary cell lines (using following primers Fw 5`-ATGGATCTCTGGCAGCTGCTGTT-3` and Rv 5`-GAAAGGCTACGGCATGATTTTGTT-3`) and cloned into pcDNA-4TO vector. CNMm-6TR cell line was subsequently transfected with pcDNA-4TO-cGHR using Lipofectamine 2000 (Invitrogen) and stably transfected cells were selected using DMEMF:12 supplemented with 10% tetracycline negative (Tet-free) FBS (PAA), 3 µg/ml blasticidin (Invitrogen) and 400 µg/ml zeocin (Invitrogen).

Transfections and luciferase assays

Transfections for luciferase assays were performed as described before[23] using 0.8 µg Spi-luc reporter plasmid[24] that is responsive to phosphorylated Stat5. In case of co-transfection with rabbit GHR (rGHR), cells were co-transfected with 10 ng pcDNA3-rGHR (gift from Prof. G.J. Strous, Utrecht Medical Center, The Netherlands). For measurement of TCF reporter activity cells were transfected with 800 ng pTOPFLASH (TOP) or pFOPFLASH (FOP) (gift from Dr. Marc van de Wetering, Hubrecht Institute, The Netherlands) and 0.5 ng human ß-actin-promoter renilla construct[25] as an internal control. Cells were left to recover for 24h in serum free medium, following by a treatment with 1 µg/ml porcine GH (Dr. A.F. Parlow, NHPP, USA) or 3 µM CHIR99021 (CHIR) (Stemgent, Germany). TCF reporter activity was calculated as an average ratio between pTOPFLASH and pFOPFLASH signal. Differences in Spi-luc and TCF reporter activities were statistically tested

using unpaired, two-tailed Student's t test in Microsoft Office Excel. All transfection experiments were performed using three replicate samples and each experiment was independently repeated 2-4 times.

RNA isolation and (quantitative) RT-PCR

RNA isolation, cDNA synthesis and (quantitative) PCR were performed as described previously[23]. Information about the primers used for (quantitative) RT-PCR is shown in the table below. Relative target gene expression was normalized to that of the reference gene RPS19 using a delta Ct method[26], and relative induction of gene expression was statistically tested using paired, two tailed Student's t test in Microsoft Office Excel. For comparison of relative gene expression over different time points, REST-MCS beta software was used (http://www.gene-quantification.de/rest-mcs.html).

Protein isolation and western blot

Protein isolation and western blotting was performed as described previously[23]. For analysis of cGHR protein induction in CNMm-cGHR, cells were seeded in 6-well plates (Primaria) at 400,000 cells/well 24h prior to 24h treatment with 1 µg/ml DOX (Sigma). Twenty µg of total cell lysates was subjected to SDS-PAGE, and analyzed by Western blot using primary antibody against GHR (T13, gift from Prof. G.J. Strous) or Pan actin (NeoMarkers/ Labvision, UK) and the secondary goat anti rabbit or goat anti mouse HRP-conjugated antibody (HAF008 or HAF007, respectively, R&D Systems). HRP was visualized using AdvanceTM Enhanced chemiluminescence (ECL, Amersham, GE Healthcare) and analyzed using GelDoc2000 (BioRad, The Netherlands).

gene	forward	reverse
Rps19	5'-CCTTCCTCAAAAAGTCTGGG-3'	5'-GTTCTCATCGTAGGGAGCAAG-3'
GH	5`-CTGCTGCTCATCCAGTCGT-3'	5`-CAGGTCCTTGAGCTTCTCGT-3`
GHR (quantitative RT-PCR)	5`-GCGCATCCCAGAGTCTACA-3`	5`-ACCATGACGAACCCCATCT-3`
GHR (full length)	5`-TATGGATCTCTGGCAGCTGCTGTT-3`	5`-GAAAGGCTACGGCATGATTTTGTT-3`
Axin2	5`-CACCCGCTCTACAACAAGGT-3`	5`-AGGTGGAGATGAAGCACAGC-3`

Results

Assessment of GH and GHR expression and signaling in canine mammary tumor cell lines

As no normal canine mammary cell lines were available to test the model of crossregulation, we initially screened a panel of canine mammary tumor cell lines for the expression of GH and GHR mRNA. All of canine mammary tumor cell lines had undetectable levels of GH mRNA (data not shown). Assessment of GHR mRNA expression showed differential levels of the transcript across the cell lines. A relatively high expression of GHR mRNA was detected in CMT1, CMT-U27, CMT9, CHMp, CNMp and CNMm cell lines (Fig. 1A). Alternative transcripts of canine GHR have previously been reported and resulted from different alternative processing of internal exons 6-9[27]. Primers used to quantify GHR transcript amplify region of exon 2 and 3 and are therefore unable to discriminate between different GHR transcripts. To check for expression of alternative GHR transcripts, PCR was performed using primers that should amplify the whole coding region of GHR. Only the full-length transcript was detected in the analyzed cell lines implying that the overall majority of transcripts are full length (Fig. 1B). Nonetheless, we were unable to detect the expression of GHR protein in the whole cell lysates of the cell lines (data not shown). To alternatively test for the expression of functional GHR protein, cell lines were transiently transfected with Spi-luc reporter that is responsive to phosporylated Stat5 protein[24] and thereby to activation of GHR. HEK cell line stably expressing rabbit GHR (HEK-rGHR) responded to treatment with exogenous GH, while none of the tested canine mammary cell lines showed enhanced Spi-luc reporter activity (Fig. 1C). Upon transient transfection with rGHR, CNMp and CNMm cell lines were able to respond to GH treatment (Fig. 1D) implicating insufficient levels of endogenous functional GHR protein in the regarding cell lines.

Assessment of crosstalk between GH and canonical Wnt signaling in canine mammary tumor cell lines expressing inducible GHR

In order to generate a canine mammary cell line model responsive to GH, CNMm cell line was stably transfected with DOX-inducible canine GHR (CNMm-cGHR). Induction of GHR mRNA and protein was detectable and stable for a period of 24 to 96 hours (Fig. 2A). Moreover, upon induction of GHR, cells were responsive to GH treatment as measured by Spi-luc reporter

Figure 1 | Canine mammary tumor cell lines have too low expression of functional GHR. (A) Rps19 normalized mRNA expression of endogenous GHR in a panel of 11 canine mammary tumor cell lines. **(B)** PCR for full-length GHR in CMT1, CMT-U27, CMT9, CHMp, CNMp and CNMm. Expected size of the full length PCR product is 1924 bp. **(C)** Fold activation of Spi-luc reporter upon treatment with exogenous GH in the cell lines expressing GHR mRNA compared to HEK-rGHR cell line. **(D)** Spi-luc fold activation in CNMp and CNMm cell lines upon transient transfection of rGHR and treatment with exogenous GH.

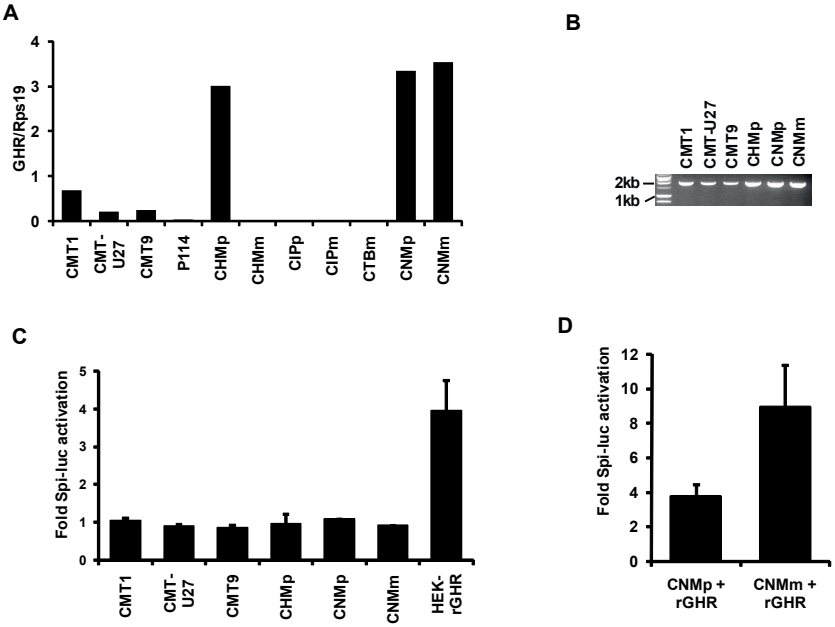


Figure 2 | Stable induction of GHR does not affect the canonical Wnt activity. (A) mRNA and protein induction of cGHR in CNMm-6TR-cGHR cells upon 0-96h DOX treatment compared to non-transfected CNMm-6TR control. Arrow indicates mature GHR protein. **(B)** Spi-luc activity in CNMm-cGHR cells upon DOX treatment in presence or absence of exogenous GH. **(C)** Average TCF reporter activity in CNMm-cGHR and CHMm-6TR cells upon DOX treatment in basal state (basal) or after GH treatment (GH). **(D)** Effect of Tet-free FBS on Spi-luc reporter activity. - indicates no DOX; + indicates DOX treatment; * indicates $p<0.05$. **(E)** Axin2 mRNA expression in CNMm-cGHR and CNMm-6TR cells upon combined DOX treatment in presence of FBS.

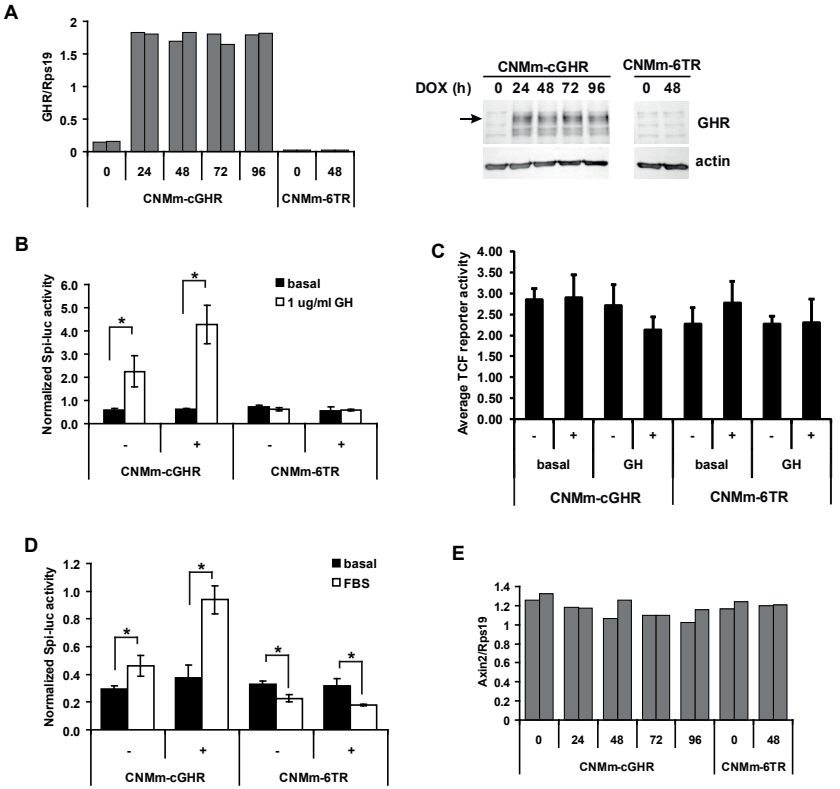
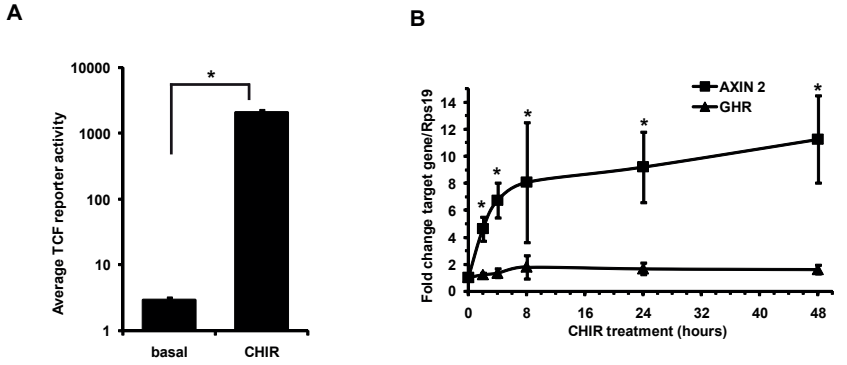


Figure 3 | Stimulation of canonical Wnt activity does not affect GHR expression. (A) TCF-reporter activity in CNMm cells upon CHIR treatment. **(B)** Fold change of Axin2 and GHR mRNA expression in CNMm cells upon CHIR treatment compared to DMSO control. * indicates p<0.05.



transactivation (Fig. 2B). Of note, even in the absence of DOX treatment transfected GHR was expressed at low levels (Fig. 2A left), sufficient to render cells responsive to GH (Fig. 2B). GHR non-transfected CNMm-6TR cells were therefore used as a negative control in the following experiments. To test whether GH signaling can influence canonical Wnt activity, CNMm-6TR and CNMm-cGHR cells were transiently transfected with TCF-reporters and treated with exogenous GH (in absence of Tet-free FBS) for 24h with or without DOX. GH treatment had no effect on TCF reporter activity in any of the conditions (Fig. 2C). To assess whether longer GH treatment is necessary to affect canonical Wnt signaling, an alternative approach was used. As prolonged removal of FBS from the medium might affect the viability of the cells, we first tested the effect of Tet-free FBS on Spi-luc reporter activity. Spi-luc reporter activity was induced by Tet-free FBS in CNMm-cGHR, but not in CNMm-6TR cells (Fig. 2D), implicating that Tet-free FBS contains sufficient levels of GH to induce GHR signaling. Axin2 is a known target gene of canonical Wnt signaling and is responsive to modulation of canonical Wnt activity in canine mammary tissue and cell lines[9]. The CNMm-6TR and CNMm-cGHR cells were therefore co-treated with Tet-free FBS and DOX for up to 96h and assessed for the expression of Axin2 mRNA. No effect on Axin2 mRNA levels was detected during this time frame. Together with

the results of TCF-reporter activity, this implicates that GH signaling cannot directly affect canonical Wnt activity in canine mammary cells.

To alternatively test whether canonical Wnt signaling is able to induce GH or GHR expression in canine mammary cells, CNMm cells were treated with a potent GSK3β inhibitor (CHIR). As expected, CHIR treatment stimulated the TCF-reporter activity and Axin2 mRNA expression (Fig. 3A and 3B). GH expression remained, however, undetectable (data not shown) and GHR mRNA expression did not change over a period of 48h (Fig. 3B).

Discussion

Oncogenic potential of progesterone in the canine mammary gland is suggested to involve induction of mammary growth hormone (GH) expression and activation of canonical Wnt signaling. In this study we tested a model in which initially progesterone-dependent induction of GH and canonical Wnt signaling involves a direct crossregulation between the two pathways. As no normal canine mammary cell lines were available to test this model, a panel of canine mammary tumor cell lines was screened for their activity and responsiveness of GH signaling. None of the tested cell lines expressed GH mRNA. This is a rather surprising observation, regarding the fact that the majority of mammary tumor tissues were shown to express GH[28]. Loss of GH expression upon generation of cell line cultures has been previously observed even in case of tumors with a high expression of GH (E. van Garderen, personal communication) and seems to be an artifact of the system. Similarly, all cell lines differentially expressed GHR mRNA, but lacked the responsiveness to GH due to insufficient levels of functional GHR protein. Additional attempts to detect unprocessed GHR protein in whole cell lysates using biotinylated human GH failed due to low affinity of human GH for canine GHR protein (data not shown). Moreover, GHR expression and signaling have previously been detected and studied in CMT-U335 cell line[29]. We additionally tested the responsiveness of this cell line using Spi-luc reporter and were unable to confirm previously published results (data not shown). The discrepancy may come from the fact that the previous study relied on immunocytochemical staining for GHR and phosphorylation of Stat5a and Stat5b upon GH treatment on western blot

of immunoprecipitated Stat proteins using general antiphosphotyrosine antibody[29].

To generate a canine mammary cell line responsive to GH stimulation, CNMm tumor cell line was stably transfected with DOX-inducible canine GHR. Using this cell line we tested whether GH signaling can influence canonical Wnt signaling by measuring TCF reporter activity or Axin2 expression upon stimulation of GHR expression by exogenous GH. Neither of the two parameters changed in the regarding cell line.

In benign mammary tissue, GH is expressed only in PR positive cells that were shown to be mainly non-proliferative[6,14]. Therefore, the expectation is that the proliferative role of GH and the crossregulation of canonical Wnt signaling in benign tissue will be through a paracrine mechanism, either direct or through induction of other paracrine factors (i.e. IGF-1). Because in the generated CNMm cell line with inducible cGHR expression, combined DOX and GH treatment did not stimulate IGF-1 expression (data not shown), we assume that the tested paracrine effects of GH in this study were direct. There have been contradictory reports of studies regarding the role of IGF-1 in regulation of canonical Wnt signaling. In some IGF-1 treatment did result in stabilization of cytoplasmic β-catenin but as a single treatment it was not able to affect TCF reporter activity[30-32]. The potential role of IGF-1 was therefore not further addressed in this study.

In tumors, however, mammary GH was suggested to contribute to oncogenic transformation of mammary epithelial cells in an autocrine fashion. Considering that the cell lines used in this study originate from tumourous tissue, effects of autocrine signaling of GH should also be tested. In a preliminary study we have transiently transfected GH into CNMm cell line stably overexpressing canine GHR and assessed its effect on TCF-reporter activity. Similarly to exogenous GH, no effect of autocrine GH production was observed on canonical Wnt activity (data not shown).

Alternatively we tested whether activation of canonical Wnt signaling using GSK3β inhibitor CHIR can affect GH or GHR expression in canine mammary cell lines, thereby stimulating the GH signaling. The treatment had, however, no effect on the expression GH or GHR. It is, however, still possible that canonical Wnt signaling is able to affect GH signaling downstream of GHR.

To test for this possibility, the effect of CHIR on Spi-luc activity should be assessed in CNMm-cGHR cells.

Altogether, based on this data, we suggest that GH and canonical Wnt signaling show no direct crossregulation in canine mammary tumors and are regulated by progesterone putatively independently of each other in the benign mammary gland. Upon activation, GH and canonical Wnt signaling may still however, act synergistically on other cellular pathways such as demonstrated for GH and non-canonical Wnt effect on EGF signaling[33]. However, to more reliably test the proposed model of crossregulation and synergism, a more representative culture system needs to be developed such as an organoid based system in which both PR positive and negative normal and/or tumorous mammary cells are present.

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CHAPTER 6

Ligand-independent canonical Wnt activity in canine mammary tumor cell lines associated with aberrant LEF1 expression

Ana Gracanin[1#], Elpetra P.M. Timmermans-Sprang[1#], Monique E. van Wolferen[1], Nagesha A.S. Rao[2], Juraj Grizelj[3], Silvijo Vince[3], Eva Hellmen[4] and Jan A. Mol[1]

[1] Department of Clinical Sciences of Companion Animals, Utrecht University, Utrecht, The Netherlands

[2] Department of Molecular Biology, Radboud University, Nijmegen, The Netherlands

[3] Clinic for Obestrics and Reproduction, Faculty of Veterinary Medicine, Zagreb, Croatia

[4] Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

[#] both authors contributed equally

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Abstract

Pet dogs very frequently develop spontaneous mammary tumors and have been suggested as a good model organism for breast cancer research. In order to obtain an insight into underlying signaling mechanisms during canine mammary tumorigenesis, in this study we assessed the incidence and the mechanism of canonical Wnt activation in a panel of 12 canine mammary tumor cell lines. We show that a subset of canine mammary cell lines exhibit a moderate canonical Wnt activity that is dependent on Wnt ligands, similar to what has been described in human breast cancer cell lines. In addition, three of the tested canine mammary cell lines have a high canonical Wnt activity that is not responsive to inhibitors of Wnt ligand secretion. Tumor cell lines with highly active canonical Wnt signaling often carry mutations in key members of the Wnt signaling cascade. These cell lines, however, carry no mutations in the coding regions of intracellular Wnt pathway components (APC, β-catenin, GSK3β, CK1α and Axin1) and have a functional β-catenin destruction complex. Interestingly, however, the cell lines with high canonical Wnt activity specifically overexpress LEF1 mRNA and the knock-down of LEF1 significantly inhibits TCF-reporter activity. In addition, LEF1 is overexpressed in a subset of canine mammary carcinomas, implicating LEF1 in ligand-independent activation of canonical Wnt signaling in canine mammary tumors. We conclude that canonical Wnt activation may be a frequent event in canine mammary tumors both through Wnt ligand-dependent and novel ligand–independent mechanisms.

Introduction

The Wnt signaling is one of the key players during normal mammary gland development as well as during mammary tumorigenesis[1]. The canonical, β-catenin-mediated Wnt signaling is activated by secreted Wnt ligands through activation of transmembrane frizzled (Fzd) receptors and LDL-receptor related protein 5 or 6 (Lpr5/6) co-receptors. This triggers Dishevelled-dependent disruption of the β-catenin-destruction complex in the cytoplasm that is composed of multiple proteins including, glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC), Axin1 (or Axin2) and casein kinase 1 (CK1). Consequently, stabilized β-catenin translocates to the nucleus where it can associate with the T-cell factor (TCF)/ Lymphoid enhancer-binding factor (LEF)-family of transcription factors to regulate the expression of specific target genes. The Wnt signal strength can be negatively regulated by secreted factors that competitively bind Wnt ligands (e.g. secreted Fzd related protein (sFRP)) or by factors that disrupt the assembly of Fzd/Lpr5/6 co-receptor complex (e.g. dikkopf-1)[2]. Many studies have documented active Wnt signaling in mammary tissue based on presence of stabilized β-catenin protein and often in combination with aberrant expression of target genes (e.g. Axin2). Based on these criteria, over 50% of human breast tumor tissue samples assessed, showed signs of aberrant canonical Wnt activity (reviewed in [3]). In contrast, reports on the signaling activity in cultured human breast cancer cells are somewhat contradicting. A number of studies have used the presence of active (phosphorylated) or uncomplexed β-catenin in cell lysates as an indicator of active canonical Wnt signaling (Table 1 and references therein). Other studies have applied a more quantitative manner to assess Wnt activity in cultured cells using a TCF-reporter assay. In this assay, the ratio of luciferase signal from reporters containing a promoter with either functional of mutated TCF response elements can be taken as a measure of canonical Wnt activity, with ratios higher than 1.0 indicating an active signaling. Although not always consistent between different studies, a subset of human breast cancer cell lines was found to have moderate TCF-reporter activity (Table 1 and references therein). In addition, a number of studies showed that the effect of canonical Wnt signaling could be attenuated by Wnt inhibitors sFRP1 or Dkk1[4,5] or by blocking the receptor Fzd7[6]. This indicates a ligand-dependent mechanism of canonical Wnt pathway activation in human breast cancer. In concordance, mutations in downstream signaling components (e.g.

Table 1 | Assessment of canonical Wnt activity in human mammary cell lines.

Cell line	Active or uncomplexed β-catenin	TCF-reporter activity (ratio >1.0)	Reference
AB589	no		4
BC3	yes		4
BT20		no	40
BT474	yes		41
BT483		no	42
BT549		no	40,42
DU4475		yes	40
EVSA-T		no	40
HCC1187		yes	25
HCC1395		yes	25
HCC1937		no	42
Hs578T		no	40
MCF7	no / yes		4/41,43
		no / yes	40/24
MDA-MB-134	no		4
MDA-MB-134VI		no	40
MDA-MB-157	yes		4
MDA-MB-175	no		4
MDA-MB-231	no /yes		41/4,43
		no / yes	25,40,44/5
MDA-MB-361	no		4
		no	42
MDA-MB-415	no		4
		no	42
MDA-MB-435	no		4
MDA-MB-453		no	40
	no		4,43
MDA-MB-468	no		4
		no	42
JIMT-1	yes		41
OCUB-F		no	40
SkBr3	no		41
SK-BR-3		no	40
SK-BR-5		no	40
SK-BR-7		no	42
SUM159		no	42
SUM185		no	42
T47D	yes		41,43

Table 1 | Continued

		no	40,44
UACC893		no	42
ZR-75-1	yes		41
		no	42

APC and β-catenin) are rarely found in human primary mammary tumors. Similarly, in cultured cells only one out of 24 screened human breast cancer cell lines (DU4475) had a truncating mutation in APC and none in β-catenin[7]. In contrast, epigenetic silencing of APC and Wnt ligand inhibitors (sFRP1 and Wif1) have often been reported in primary human mammary tumors and in human breast cancer cell lines[8-10]. The ligand-dependent nature of canonical Wnt activation in human breast cancer and benign breast lesions is further supported by frequent overexpression of Wnt ligands[3]. Pet dogs have been suggested as a valuable breast cancer model for preclinical research due to the high incidence and spontaneous nature of the tumor development, shared environmental risk factors, strong genetic similarity with humans and shared aspects of mammary tumor biology[11,12]. Moreover, development of canine mammary tumors is highly dependent on steroid hormone exposure, with progesterone being the main risk factor[13,14]. The activity of canonical Wnt signaling in canine mammary tumorigenesis has not been quantitatively assessed so far. Previous studies have only addressed the expression of β-catenin protein in spontaneous canine mammary tumors in relation to E-cadherin and/or APC[15-17]. However, comparative gene expression profiling of human and canine mammary tumors has implicated a significant similarity in deregulation of multiple cancer-related pathways, including Wnt signaling[18]. In this study we aimed to assess the activation of canonical Wnt signaling in canine mammary tumors using a panel of canine mammary cell lines. We report that subsets of canine mammary tumor cell lines exhibit moderate, ligand-dependent-, and high, ligand-independent-mechanisms of canonical Wnt activation. Moreover, we show that the ligand-independent activation of canonical Wnt signaling is coupled to the overexpression of LEF1.

Materials and Methods

Canine mammary cell lines and tissue

Canine mammary tumor cell lines used in this study were established from primary tumors diagnosed as carcinoma (CMT1, CMT-U27, CMT9, P114, CHMp, CNMp and CIPp) or its metastasis (CHMm, CNMm and CIPm), benign mixed tumor (CMT-U229) and osteosarcoma-like tumor (CMT-U335)[19-21]. All cell lines were cultured in DMEM/F12 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (FBS Gold, PAA, Cölbe, Germany). Canine mammary tissue used in this study originates from privately owned dogs that were referred to clinics of Veterinary Faculty in Zagreb, Croatia. Canine mammary surgery was performed as a part of a necessary medical treatment due to the presence of mammary tumor. This was done under the common rules for veterinary surgery for which owners asked for medical treatment of their pets. In contrast to medical intervention in laboratory animals no external permission was necessary other than that the surgery is done by qualified veterinary surgeons. The dog's owners were informed and gave their consent that the collected tissues can be used for research purposes. Histopathology of all tumor and paired normal tissue was evaluated by Prof. E. Hellmen (Table 2). Pictures of cell morphology were captured using an Olympus microscope (Zoeterwoude, The Netherlands) with 10x10 magnification.

TCF-reporter assay

Transfection was performed in FBS-free medium using 3 µl Lipofectamine 2000 (Invitrogen), 800 ng pTOPFLASH (TOP) or pFOPFLASH (FOP) (gift from Dr. Marc van de Wetering, Hubrecht Institute, The Netherlands) and 0.5 ng human ß-actin-promoter renilla construct[22] as an internal control. Cells were seeded 48h before transfection at a density optimal for transfection, according to the manufacturer's protocol in a 24 wells plate (Primaria, BD Biosciences, Breda, The Netherlands). In case of Wnt3a cotransfection, 10 ng mouse pcDNA4-Wnt3a construct (gift from Dr. Wim de Lau, Hubrecht Institute, The Netherlands) was used. Transfection was stopped after 5h and cells were left to recover for 24h on DMEM/F12 supplemented with 10% FBS. Cells were then treated with increasing concentrations of IWP-2 (Stemgent, Cambridge, UK) or 5 mM LiCl for 24h. Control DMSO concentration reflected the DMSO concentration in the 10 µM IWP-2 solution. The firefly and renilla luciferase activities were measured using a Dual-Luciferase Assay System (Promega, Leiden, The Netherlands) in a Centro LB 960 luminometer

Table 2 | Information about histopathology and RNA quality of canine mammary tumor tissue

Sample ID	Tumor histopathology	Normal (RIN)	Tumor (RIN)
2	Benign mixed tumor	8.8	8.1
3	Complex adenoma	7.7	8.4
7	Complex adenoma	8.3	8.6
14	Carcinosarcoma (combined osteosarcoma and ductular carcinoma)	7.9	8.3
20	In situ carcinoma	7.6	7.9
25	Atypical sclerosing adenosis and purulent inflammation	7.8	9.2
26	Simple solid carcinoma	8.9	9.0
1	Simple ductal carcinoma	7.1	9.3
5	Simple carcinoma	6.5	8.0
24	Complex carcinoma	8.4	9.8
31	Probable fibrosarcoma / complex carcinoma	8.6	7.1

Table 3 | Information about primers used to assess gene expression

Gene name	Forward primer	Reverse primer	Annealing T (°C)
SFRP1	AGCGAGTTTGCATTGAGGAT	TCTTGATGGGTCCCAACTTC	60
APC	AGTCCCAAGCAACAGAAGC	GCAGTTGAACCCTGAGCA	63
β-catenin (CTNNB1)	ATGGGTAGGGCAAATCAGTAAGAGGT	AAGCATCGTATCACAGCAGGTTAC	64
E-cadherin (CDH1)	CAGGAAGCTCTCCACCAGAG	CTGGGAAATGTGAGCACCTC	58
LEF1	AGACATCCTCCAGCTCCTGA	GATGGATAGGGTTGCCTGAA	60
TCF1 (TCF7)	CTACTCCGCCTTCAATCTGC	AGAGAGTTGTGGGACGCTGT	60
TCF3 (TCF7L1)	CCTGGAGCTGTTGGACAAAT	AAACCAGGCTGGACATTGAG	60
TCF4 (TCF7L2)	CGAGTGCACGTTGAAAGAAA	ATGTGAAGCTGTCGCTCCTT	60
WNT1	CTGGCACGTTGACTCAGAGA	AAGAGCTGCATAGCCACCAC	63
WNT2	GACAGGGATCACAGCCTCTT	TGGTGATGGCAAACACAACT	63
WNT3	ATGAACAAGCACAACAACGAG	TTGAGGAAGTCGCCGATAG	61.5
WNT4	CGAGGAGTGCCAGTACCAGT	AGAGATGGCGTACACGAAGG	61,6
WNT5B	CCCTGTACAGAGACCCGAGA	ACAACTGGCACAGCTTCCTC	61.5
WNT7A	GCCTCGACGAGTGTCAGTTT	GATGATGGCGTAGGTGAAGG	60
RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	61
AXIN2	GGACAAATGCGTGGATACCT	TGCTTGGAGACAATGCTGTT	60

(Berthold Technologies, Vilvoorde, Belgium). Differences in pTOPFLASH/pFOPFLASH were statistically assessed using unpaired, two tailed Student's t test in Microsoft Office Excel. All transfection experiments were performed using three replicate samples and each experiment was independently repeated 2-4 times.

siRNA

Canine sequence-specific LEF1 (synonym: TCF1-alpha, Genbank: XM_003434032) and β-catenin (CTNNB1, Genbank: NM_001137652) siRNA was designed on the website http://www.dharmacon.com/designcenter/designcenterpage.aspx (DharmaconRNAi technologies, ThermoScientific, USA). Universal MOCKsiRNA (ON-TARGET plus non-targeting pool species H, M, R) was used as the negative control for siRNA experiments. There was no cross-silencing of non-target genes checked by blasting the siRNA designed sequences against the canine genome database. The sequence of the LEF1 siRNA duplex is as follows: sense GAAGAGGAAGAGAGAGAAAUU and antisense UUUCUCUCUCUUCCUCUUCUU, and for β-catenin sense GAACGAAGGUGUAGCAACAUU and antisense UGUUGCUACACCUUCGUUCUU. Cell transfections were first optimized with siGLO (Dharmacon, Colorado) (data not shown). 80.000 CMT-U27 cells were transfected with 1 µl DharmaFECT Duo as transfection reagent (Dharmacon, Colorado), 50 nM siRNA and 0.5 µg DNA (TOP or FOP) in 24 wells plates (Primaria, BD, The Netherlands). After 24h and 48h incubation in DMEM:F12 and 10% FCS cells were harvested for RNA isolation or TCF-reporter assay.

RNA isolation, cDNA synthesis, sequencing and quantitative RT-PCR

From all cell lines, total RNA was isolated from two different passages. From canine mammary tissue and from the mammary cell lines total RNA was isolated and treated with deoxyribonuclease using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Quality of mRNA from tissue samples were assessed using a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) and RNA integrity number (RIN) of each sample is presented in Table 2. cDNA synthesis was performed using iScript kit (Bio-Rad Laboratories) according to manufacturer's protocol. Specific primer sets were used to amplify gene products for quantitative RT-PCR (Table 3) and sequencing (Table 4). Quantitative RT-PCR was performed using Bio Rad MyIQ detection system

(Bio-RAD Laboratories) with SYBR Green Fluorophore. Relative target gene expression was normalized to that of the reference gene RPS19 using a delta Ct method[23], and relative induction of gene expression was statistically assessed using paired, two tailed Student's t test in Microsoft Office Excel. For comparison of relative gene expression between three sets of cell line, REST-MCS beta software was used (http://www.gene-quantification.de/rest-mcs.html). For the sequence reactions we used a standard amplification with Phusion-Hot Start Taq (Finnzymes, Espoo, Finland) according to manufacturer's protocol. DNA sequence reactions were performed using BigDye v3.1 according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). All amplifications were performed on an ABI 3130XL (Applied Biosystems, Foster City, CA) and analyzed in Lasergene (version 9.1 DNASTAR). The obtained sequences were compared with DNA sequences in databases using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 4 | Information about primers used to sequence gene coding regions

gene name	Forward primer	Location	Reverse primer	Location
β-catenin	AAGCACACCATACAACGG	F4	CCTAAACCACTCCCACCCT	R2494
(CTNNB1)	GGCTGCTATGTTCCCTGAGA	F327	CCACCTGGTCCTCATCATTA	R541
	GGGACCTTGCACAATCTTTCTC	F688		
	AATGCAGGCTTTAGGGCTTCA	F1128		
	CCTGCCATCTGTGCTCTTCGTC	F1429		
	TCACAACCGAATCGTAATCAGA	F1794		
APC	AGAGGCAGACTCAGCACCAT	F3728	GGGGCTTATAATGCCACTCA	R4311
	GGCATTATAAGCCCCAGTGA	F4297	ACAGGGGGAGGTAATTTTGG	R4865
	AAAGAGCCCGAAAAGCCTAC	F4702	ACACGGAAAGGCTTGTGACT	R5279
	GCCCAAAGGAAAAAGTCACA	F5247	CGATTTACGGGGTGTTTTGT	R5810
	CCAGGGAAAAGGCTGAATTA	F5675	ACTCCTGCAACAGGTCGTCT	R6178
	AGACGACCTGTTGCAGGAGT	F6159	GGGCTGTTTCATTTGGCTTA	R6702
	AGCAAACATGCCTTCGATCT	F6708	CCTTTGGAGGCAGACTCACT	R7253
	ACGTCTCCAGGCAGACAGAT	F7153	GAATGGGAGCGTGCAATATC	R7652
	CACGCTCCCATTCTGAAAGT	F7640	CCGTTACCCACACTTGGTTT	R8180

Table 4 | Continued

	GAGATCCCCAACAGGAAACA	F8070	CACACGGATGTCACGAGGTA	R8585
GSK3B	GAGGGTGATTCGGGAAGAG	F1005	TAGGCTAAACTTCGGAACAG	R1542
	CGGAAACAGTATACAGAGTTGC	F1437	AAGTAACTGGTGGTTCTTCCTG	R2338
	TTCCCTCAAATTAAGCAC	F1907		
CK1	GAGCGGCGGCGATCAGGTTCC	F217	ATACCCATTAGGAAGTTATCTGGT	R835
(CSNK1A1)	TACACAGAGACATTAAACCAGATA	F796	ATCTGCTCTGCTTCTTCTGTTC	R1446
	ATAAAAATCTCACTGGCACTG	F1016		
AXIN1			CTTCGTTCAGAGTGGGCAGGTAGC	R730
	GTTTGACCAGGCACAGACGGAGAT	F543	CGGTAAGTGCGAGGAATGTGAGGT	R1127
	ACCGACAGCAGCGTGGAT	F1006	ACTGTGGTGACTGTGGTGGTG	R1626
	CCGGCCATCGTTCCCCTGACAAT	F1466	GCGGTGCCTGCTGATCTCCTTCTC	R1941
	TCGGAGGACACAGACAAGAAC	F1867	GAACCTCCTCGAACACCACTC	R2536
	GCGGAGTGGTGTTCGAGGAGGTTC	F2513		
E-cadherin			TCTGGTTTATGAAGTTGTAGAGGC	R399
(CDH1)	CAGCCTATGTTTCTGATGAC	F312	ACAGATCCTTGGAAGACTGC	R921
	AGTCTTCCAAGGATCTGTCAC	F904	CAGTCACGTACAAGACATACTG	R1475
	TTTGTTGTCACCACAGACCC	F1376	CAATGATGTTGATGACATGAGG	R1955
	ACCTCGAAATATGGACTTCTG	F1897	CAACTGGCTCAAGTCAAAGTC	R2428
	CTCGCTCTACTAATCCTGATTCTG	F2264	CCATTCATTCAGGTAGTCATAGTC	R2707
			TTCTCCAGAAATTCCTCTCAG	R2861
LEF1	GAGCGGAGATTGCAGAGC	F615	CGTTGGGAATGAGTTTCGTT	R1875

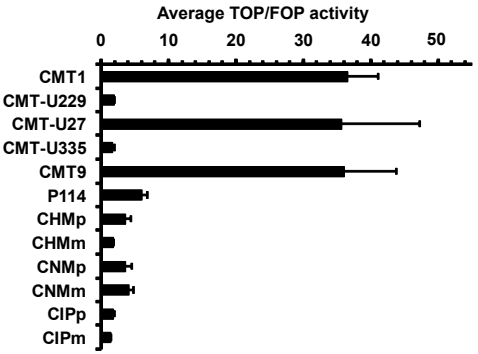


Figure 1 | TCF-reporter activity in canine mammary tumor cell lines. Average TOP/FOP ratio (+/- s.e.m.) in canine mammary cell lines from 3-4 independent experiments.

Protein extraction and Western blot

For whole cell lysis cells were washed with cold HANK's balanced salt solution and scraped in cold RIPA buffer (6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl (pH 7.4); 1% sodium dodecyl sulfate (SDS) (vol/vol), 1% Igepal (vol/vol), 0.5% Na-deoxycholate (wt/vol), 1 mM phenylmethylsulfonylfluoride,1 mM Na-orthovanadate and 1 µg/ml aprotinin). After 20 min incubation on ice, samples were centrifuged for 15 min at 16,000g at 4°C. Protein concentration was determined using Bio-Rad Dc Protein Assay (Bio-Rad Laboratories). Fifty microgram protein of total cell lysates was subjected to SDS-PAGE and analyzed by Western blot. For extraction of cytoplasmic and nuclear protein fractions NE-PER Reagent kit (Thermo Scientific, Breda, The Netherlands) was used according to the manufacturer's protocol. Ten µg protein was subsequently subjected to western blot analysis. Primary antibodies used in this study were directed against APC (AB-1) Mouse (FE9) (OP44 1:1000), (Calbiochem, Merck, Amsterdam, The Netherlands), β-catenin (Ab6302 1:4000)(Abcam, Cambridge, UK), human E-cadherin (610181, 1:2000 BD Biosciences, Breda, The Netherlands), GAPDH (ab9485 1:2000) (Abcam) and β-actin pan Ab-5 (MS-1295-P1 1:2000)(Thermo Scientific) as a reference protein. And as secondary antibody goat anti-mouse HRP-conjugated (HAF007, R&D Systems, Abingdon, UK) was used. HRP was visualized using Advance TM_ Enhancedchemiluminescence (ECL, Amersham, GE Healthcare, Eindhoven, The Netherlands) and analyzed using GelDoc2000 (BioRad).

Results and Discussion

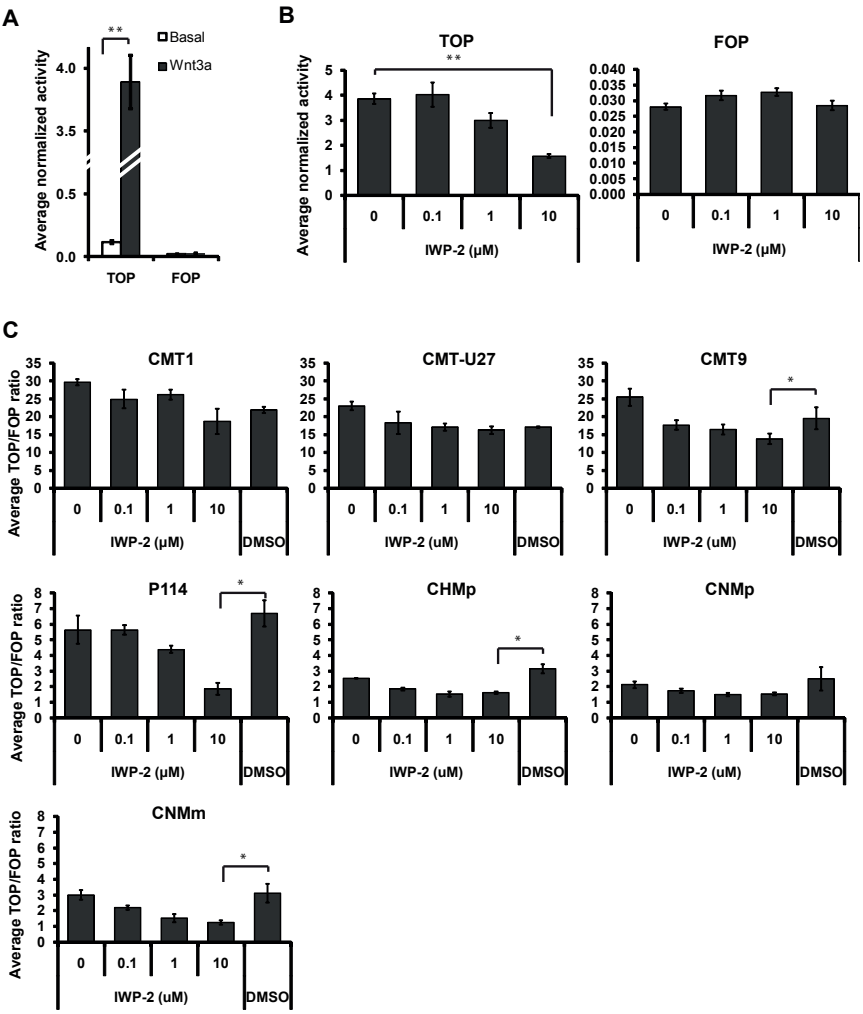
Canonical Wnt signaling is aberrantly active in a subset of canine mammary tumor cell lines

To quantitatively assess canonical Wnt activity we tested a panel of 12 canine mammary cell lines using a TCF-reporter assay (Fig.1). Three cell lines (CMT1, CMT-U27 and CMT9) showed high TCF-reporter activity. Four cell lines (P114, CHMp, CNMp and CNMm) showed moderate reporter activity comparable to previously reported activity in human mammary cell lines[24,25]. The remaining five cell lines (CMT-U229, CMT-U335, CHMm, CIPp and CIPm) with the TOP/FOP ratio around 1, lacked canonical Wnt activity.

Ligand-dependent and -independent mechanism of canonical Wnt activation in canine mammary cell lines

IWP-2 is a small molecule inhibitor that impairs Wnt ligand palmitoylation and secretion[26] and could, therefore, be used to discriminate between ligand-dependent and -independent mechanisms of canonical Wnt activation in cells. To assess the activity of IWP-2 in canine cells, P114 cell line was transiently co-transfected with canonical Wnt ligand Wnt3a (Fig. 2A) and treated with increasing concentration of IWP-2. IWP-2 effectively inhibited Wnt-3a-dependent TOP-flash activity, but not the FOP-flash activity (Fig. 2B), confirming its specificity as canonical Wnt inhibitor in canine cells. The effect of IWP-2 treatment on the basal canonical Wnt activity was subsequently evaluated in all cell lines with active Wnt signaling. In cell lines with moderate basal canonical Wnt activity (i.e. P114, CHMp, CNMp and CNMm), IWP-2 was able to efficiently inhibit the TCF-reporter activity. Treatment with 10 µM IWP-2 resulted in TOP/FOP ratios around 1, suggesting a full ligand-dependency in the cell lines with moderately activate Wnt signaling (Fig. 2C). Moreover, we have assessed the expression of several Wnt ligands previously reported as activators of canonical signaling and/or being expressed in mammary tissue and cell lines[27,28]. Ligand-dependent activation of the pathway in these cell lines is further supported by the high expression of multiple Wnt ligands (especially Wnt5b and Wnt7a) and undetectable levels of the inhibitor sFRP1 (Fig. 3). IWP-2 treatment in CMT1, CMT-U27 and CMT9 cells, however, had no or only a minor effect on the TCF-reporter activity (Fig. 2C). These three cell lines are therefore expected to have a ligand-independent component for canonical Wnt activation. Recently, it has been reported that conditioned medium of tumor-associated macrophages or co-culture with macrophages mediate a switch from canonical to non-canonical Wnt signaling in multiple canine mammary cell lines, including P114 and CMT-U27[29]. Inhibition of canonical Wnt signaling (demonstrated by downregulation of cytoplasmic and nuclear ß-catenin protein levels) was associated with exposure of cells to increased levels of non-canonical Wnt ligands and canonical Wnt inhibitor Dkk-1. Our data supports the responsiveness of P114 cell line to Wnt ligands and inhibitors. However, the insensitivity of basal TCF-reporter activity in CMT-U27 cells to treatment with IWP-2 suggests that the reported inhibition of canonical Wnt signaling[29] is not mediated by altered Wnt ligand or inhibitor expression, but most probably is caused by other mechanisms.

Figure 2 | Inhibition of canonical Wnt activity using porcupine inhibitor IWP-2. (A) Effect of transient Wnt3a co-transfection on TOP and FOP activities in P114 cells. **(B)** Effect of IWP-2 treatment on TOP and FOP activities in P114 cells co-transfected with Wnt-3a. **(C)** Effect of IWP-2 treatment on basal TOP/FOP ratio in CMT1, CMT-U27, CMT9, P114, CHMp, CNMp and CNMm cell lines. TOP/FOP ratio after treatment with 10 µM IWP-2 was tested against the control DMSO treatment. * indicates p<0.05 and ** p<0.01.



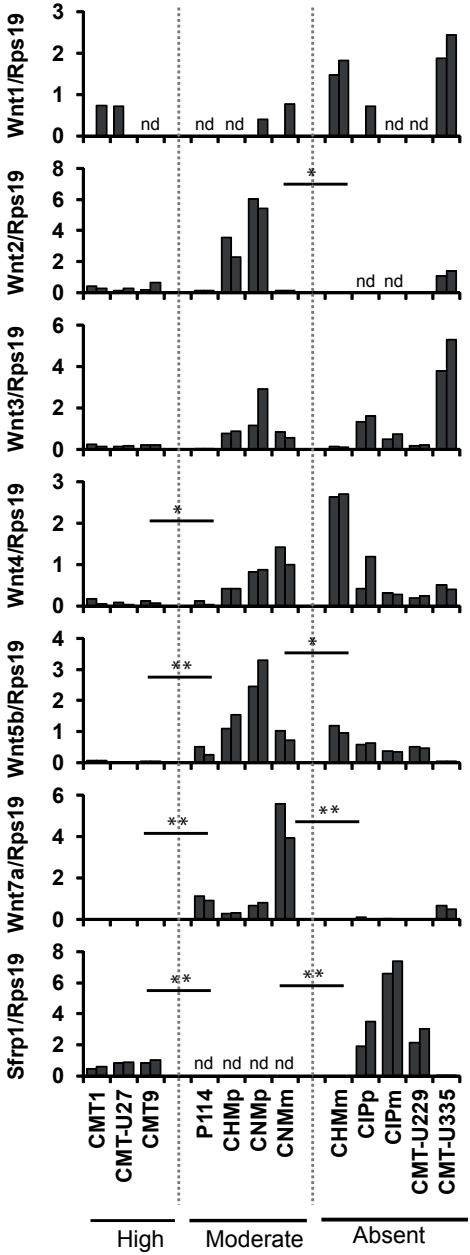
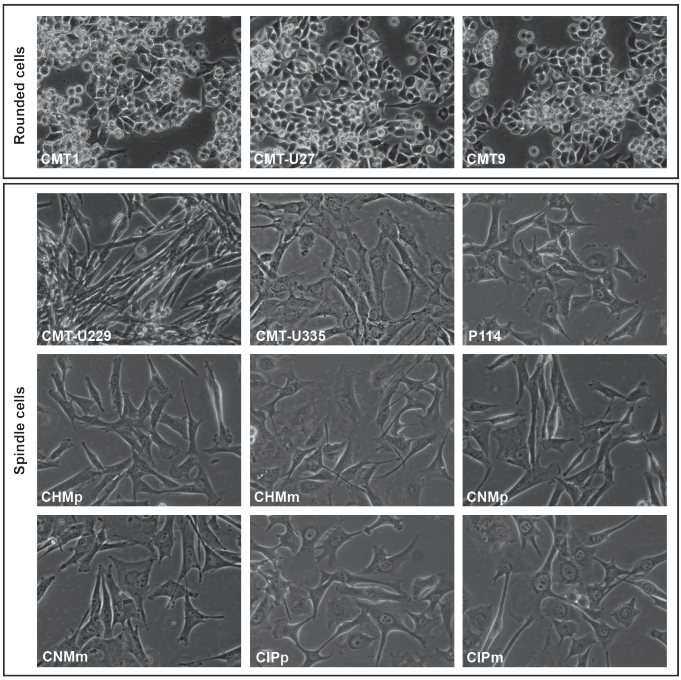


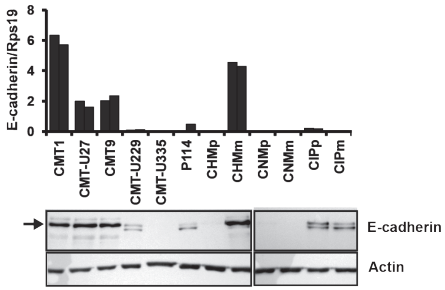
Figure 3 | Expression of multiple Wnt ligand- and inhibitor Sfrp1-mRNA. mRNA expression of Wnt1, Wnt2, Wnt3, Wnt4, Wnt5b, Wnt7a and Sfrp1 in two different passages of cell lines. Target gene expression was normalized to that of a reference gene Rps19. Cell lines were divided in three groups (from left to right): cell lines with high, moderate or absent canonical Wnt activity. * indicates $p<0.05$, ** $p<0.01$ and nd stands for non-detectable.

Figure 4 | Cell line morphology and E-cadherin expression. (A) Canine mammary cell lines grouped based on their morphology as (partially) rounded cells or spindle cells. **(B)** Expression of E-cadherin at mRNA (top) and protein (bottom) level. Rps19 and actin expression served as reference mRNA and protein, respectively. Arrow indicates the position of full-length mature E-cadherin protein. Additional E-cadherin protein band present in some of the cell lines represents the unprocessed form of the protein.

A



B



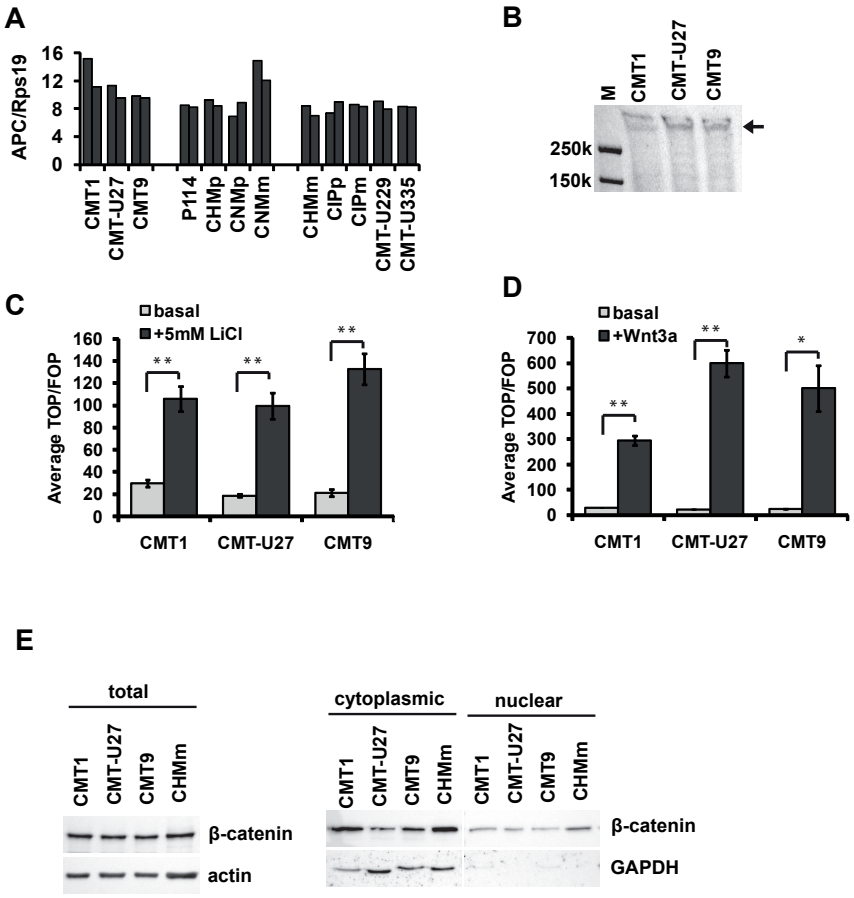
High canonical Wnt activity is not associated with a lack of functional E-cadherin

The majority of the canine mammary cell lines used in this study had spindle-cell-like morphology, except for CMT1, CMT-U27 and CMT9, which grow as attached cells but also as partially rounded cells (Fig. 4A). A partially rounded morphology has been associated with E-cadherin mutations in human breast cancer cell lines[30]. As the loss of E-cadherin protein can stimulate canonical Wnt activity[31], we analyzed its coding sequence and mRNA and protein levels in all 12 canine mammary cell lines. Sequencing analysis of the whole CDH1 coding region revealed no mutations in any of the cell lines (Table S1). Moreover, CMT1, CMT-U27 and CMT9 highly expressed mRNA and mature protein of E-cadherin (Fig. 4B), suggesting a different mechanism of canonical Wnt activation in these cell lines.

High canonical Wnt activity is not associated with defects in β-catenin destruction complex

In multiple tumors, elevated canonical Wnt activity has been shown to result from mutations in components of β-catenin destruction complex[32]. Mutational analysis of coding sequences of APC, β-catenin, GSK3β, CK1α and Axin1 in canine mammary cell lines revealed, however, no mutations that were restricted to cell lines with the active canonical Wnt signaling (Table S1). As APC is also known to be epigenetically silenced or proteolytically cleaved in tumors[33,34], its mRNA and protein expression were additionally assessed. All 12 canine mammary cell lines expressed comparable levels of APC mRNA (Fig. 5A). Analysis of protein expression in CMT1, CMT-U27 and CMT9 revealed that APC was expressed as a full-length protein (Fig. 5B). We next asked whether high canonical Wnt activity in these cells is a consequence of a defect at the level of β-catenin destruction complex. Canonical Wnt signaling in cells in which β-catenin destruction complex function is fully impaired is expected to be insensitive to further stimulation of the pathway by Wnt ligands or to treatment with GSK3β inhibitors[35]. CMT1, CMT-U27 and CMT9 cells, however, responded potently to GSK3β inhibitor, LiCl (Fig. 5C) as well as to Wnt3a transfection (Fig. 5D). To determine whether the high Wnt activity is associated with increased stabilization of β-catenin protein we assessed total, cytoplasmic and nuclear levels of β-catenin by western blot. GAPDH was used as a marker of cytoplasmic proteins to assess the purity of extracts from different cell fractions. When compared to a cell line lacking canonical Wnt activity (CHMm), CMT1, CMT-U27 and CMT9 cells

Figure 5 | Assessment of defects at the level of β-catenin destruction complex.(A) mRNA expression of APC in two different passages of canine mammary cell lines normalized to the expression of Rps19. **(B)** APC protein expression in CMT1, CMT-U27 and CMT9. M indicates loading marker with reference molecular weight bands. **(C)** Effect of treatment with 5mM LiCl or co-transfection with Wnt-3a **(D)** on TCF-reporter activity in CMT1, CMT-U27 and CMT9. * indicates $p<0.05$ and ** $p<0.01$. **(E)** β-catenin protein expression in total cell lysates (total) and the cytoplasmic and nuclear fractions.

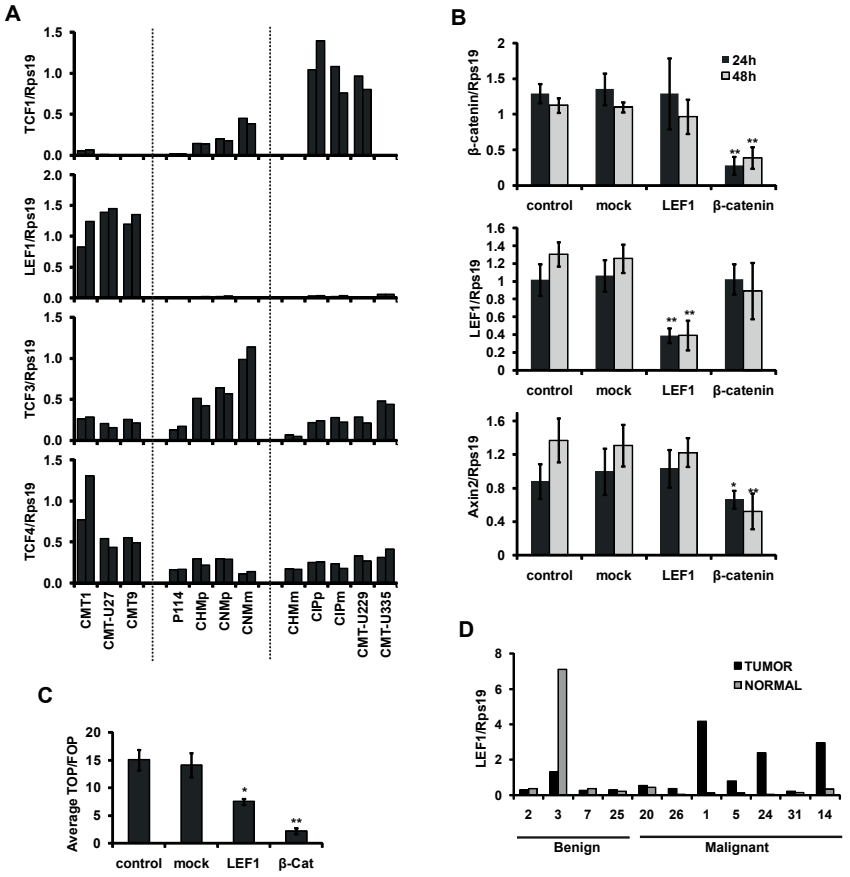


did not show evidence of marked cytoplasmic or nuclear β-catenin protein stabilization (Fig. 5E), implying no major defect in the β-catenin destruction complex function.

Ectopic LEF1 expression contributes to the high canonical Wnt activity in CMT1, CMT-U27 and CMT9

Upon stabilization, β-catenin translocates to the nucleus and interacts with members of TCF/LEF family of transcription factors (TCF1, LEF1, TCF2 and TCF4). Interestingly, although all TCF/LEF family members were expressed in canine mammary cell lines, LEF1 showed high mRNA expression specifically in CMT1, CMT-U27 and CMT9 (Fig. 6A). Different LEF1 isoforms have been described, resulting from alternative splicing of LEF1 transcript[36]. Sequencing of LEF1 coding region in CMT1, CMT-U27, CMT9 and CIPp showed that the first three cell lines express LEF1 transcript lacking exon 6. Lack of exon 6 in Xenopus LEF1 has been shown to lower its transcriptional potential on TOPFlash reporter in HEK293 cells[37]. Lack of exon 6 in LEF1 in CMT1, CMT-U27 and CMT9 can therefore not be attributing to the high canonical Wnt activity in these cell lines. As LEF1 is known to be a direct target gene of canonical Wnt signaling[38] we asked whether high expression of LEF1 in these three cell lines could be a cause or a consequence of high canonical Wnt activity. For this purpose β-catenin and LEF1 knock down was performed in CMT-U27 cells. Knock-down of β-catenin resulted in a potent inhibition of TCF-reporter activity (Fig. 6C) and down regulation of Axin2 target gene (Fig. 6B), but it had no effect on the expression of LEF1 (Fig. 6B). Knock-down of LEF1 did not affect β-catenin expression (Fig. 6B) but was able to significantly inhibit TCF-reporter activity (Fig. 6C). Altogether, this suggests that the ectopic expression of LEF1 in CMT1, CMT-U27 and CMT9 is not a consequence but rather a contributing factor to the high canonical Wnt activity in these cell lines. LEF1 was recently shown to affect the viability, invasion and migration of breast cancer cells[39]. The correlation between LEF1 overexpression and high canonical Wnt activity in canine mammary tumor cell lines prompted us, therefore, to assess LEF1 expression in spontaneous canine mammary tumors. To assess the tumor-specific overexpression of LEF1, each canine mammary tumor was compared to the corresponding normal tissue from the same patient. Interestingly, five out of seven malignant tumor samples showed overexpression of LEF1 (Fig. 6D). However, the sample size will need to be increased in order to test the statistical significance of these results. A challenging task remains to assess

Figure 6 | Association between high canonical Wnt activity and LEF1 expression. (A) mRNA expression of TCF1, LEF1, TCF3 and TCF4 in two different passages of canine mammary cell lines normalized to the expression of Rps19. **(B)** β-catenin, LEF1 and Axin2 mRNA expression in CMT-U27 cells that were either non-transfected (control) or transfected with mock control (mock), LEF1 siRNA (LEF1) or β-catenin siRNA (β-catenin). mRNA expression was analyzed 24h and 48h post-transfection. **(C)** Average TOP/FOP ratio in CMT-U27 cells as described in **(B)**. * indicates p<0.05 and ** p<0.01 compared to the mock control. **(D)** Rps19 normalized mRNA expression of LEF1 in a panel of canine mammary tumors (tumor) and normal mammary tissue (normal) from the same dog.



whether LEF1 overexpression in canine mammary tumors is associated with high canonical Wnt activity. Considering that canine mammary tumor cell lines with high LEF1 expression do not seem to show marked overstabilization of β-catenin protein (Fig. 5E), an alternative marker for canonical Wnt activity in tissue samples is needed. In this regard, Axin2 mRNA levels were suggested to correlate with mutations in the Wnt signaling pathway in a panel of human cancer cell lines[32]. However, in canine mammary cell lines, basal Axin2 mRNA levels do not correlate with the canonical Wnt activity (Fig. S1), implicating that Axin2 expression is also not a reliable canonical Wnt activity marker in canine mammary tumors. On a further note, LEF1 knock-down was not able to fully inhibit TCF-reporter activity in canine mammary cell lines. This may be a consequence of insufficient knock-down of LEF1 mRNA but it may also argue for involvement of additional canonical Wnt activating factors. To test whether other TCFs may be compensating for the knock-down of LEF1, the levels of all TCF-family members were assessed 24h after knock-down of LEF1 (Fig. S2). Expression of neither of TCFs showed, however, signs of compensation. Lastly, the use of transient transfection system prevented us from investigating a relationship between LEF1 overexpression and the cellular morphology. For this purpose stable transfection of inducible LEF1 knock-down system should be employed.

Conclusion

Altogether, this study provides evidence for moderate, ligand-dependent canonical Wnt activation in canine mammary tumors that is comparable to human breast cancer. In addition, we report a novel ligand-independent mechanism involving LEF1 overexpression, which results in high canonical Wnt activity. Our further studies aim to explore this ligand-independent mechanism extensively and to identify the underlying gene mutations.

Supplementary Figure legends and Tables

Figure S1 | Axin2 mRNA expression. Rps19 normalized Axin2 mRNA expression in two different passages of canine mammary cell lines. Cell lines were divided in three groups (from left to right): cell lines with high, moderate or absent canonical Wnt activity.

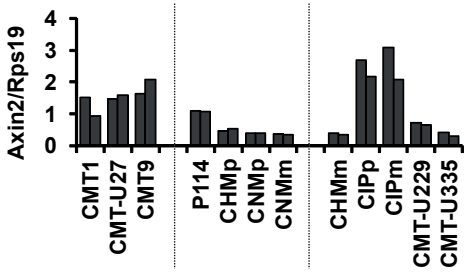


Figure S2 | Expression of TCF-family members upon LEF1 knock-down. Relative Rps19 normalized mRNA expression of LEF1, TCF1, TCF3 and TCF4 24h after LEF1 knock-down in CMT-U27 cells. Average expression of control conditions for each target gene is set to 100.

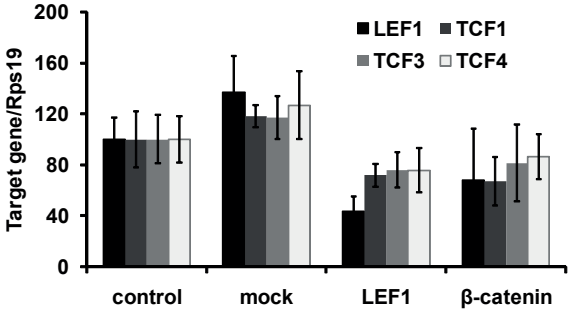


Table S1 | Sequencing results of target gene coding regions in canine mammary tumor cell lines.

Transcript	β-catenin	APC*	GSK3β	Axin1	CK1α	CDH1	LEF1
ref seq	NM_001137652.1	XM_536285	XM_851518.2	XM_847228	XM_536470	XM_536807	XM_858241
CMT1	wt	wt	snp 1807 GGG->GGA	50% snp 1753 GCA->GCG snp 2344 GCC->GCT snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	wt	snp1743 ACG>ACA	Missing exon 6
CMT-U27	wt	wt	snp 1807 GGG->GGA	50% snp 1753 GCA->GCG snp 2344 GCC->GCT snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	wt	snp1743 ACG>ACA	Missing exon 6
CMT9	wt	wt	snp 1807 GGG->GGA	50% snp 1753 GCA->GCG snp 2344 GCC->GCT snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	wt	snp1743 ACG>ACA	Missing exon 6
P114	wt			snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)		snp1743 ACG>ACA	
CHMp	50% snp 1770 GCC->GCT 50% snp 2134 CTT->ATT (L->I)					snp1743 ACG>ACA	
CHMm	50% snp 1770 GCC->GCT 50% snp 2134 CTT->ATT (L->I)					snp1743 ACG>ACA	
CIPp	wt			snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	wt		wt

Table S1 | Continued

ClPm		snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	snp1743 ACG>ACR
CNMp	50% snp 1770 GCC->GCT 50% snp 2134 CTT->ATT (L->I)	snp 2550+2551 TCA->TTC (S->F) snp 2658…2666 ACA GGT GGA TGA-> AAG GTG GAC TGA (T G G - -> L V D -)	
CNMm	66%snp 1770 GCC->GCT		

*mutation cluster region and the downstream sequence

wt = wild-type, SNP = single nucleotide polymorphism, underlined = changes affecting protein sequence

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CHAPTER 7

Summarizing discussion

Breast cancer is the most common type of malignancy in women for which ovarian hormones were identified as an important risk factor. More specifically, progesterone and its downstream signaling mechanisms are implicated as important players in the process of mammary tumorigenesis[1,2]. An important aspect in this regard is that progesterone signaling can play a role during tumor initiation as well as progression. It is therefore important to choose a suitable model system that could be used to address separately both the initiation and the progression phases. Dogs make an appropriate model in this aspect because of the high progesterone-dependent incidence of mammary tumors. Moreover, there is a large availability of established canine mammary cell lines, while canine mammary (tumor) tissue can easily be obtained from patients referred to veterinary hospitals. Nonetheless, the mechanisms behind progestin-induced mammary tumorigenesis in dogs have remained largely unexplored and no studies have addressed progesterone signaling in dogs since the cloning of canine progesterone receptor (PR) sequence[3].

In this thesis we first addressed the role of progesterone in canine mammary tumor initiation by studying the function and expression of canine PR. PR is expressed as two main protein isoforms, PR-B and a truncated PR-A protein. In **Chapter 2** we report an intriguing finding regarding the function of canine PR-B isoform. Unlike other mammals, dogs have accumulated multiple mutations in the AF3 domain that is essential for the transcriptional activity of PR-B. As a consequence, canine PR-B has a very limited transcriptional activity as evidenced by low transactivation of progestin-responsive luciferase reporters and endogenous target genes. These results were confirmed and even more striking in **Chapter 3** were we performed expression profiling using dog-specific microarray on canine mammary tumor cell lines expressing inducible human PR-B, canine PR-B or a chimera of canine PR-A and human AF3 domain. In contrast to human and chimera PR-B, canine PR-B regulated expression of only a small subset of genes and to a very limited extent thereby questioning its biological relevance. In **Chapter 2** is, furthermore, shown that the phenomenon of poor AF3 domain-dependent transcriptional activity appears unique for canids (wolf- and fox-like species) and correlates strikingly with their unusual progesterone-related reproductive biology. This includes prolonged luteal phase of estrous cycle often associated with pseudopregnancy and in some species alloparenty (where non-reproductive females are able to lactate and nurture offspring

of reproductive females)[4]. Moreover, in **Chapter 4** we generated new canine PR-B-specific antibody and showed that the expression of canine PR-B is either very limited or absent in the mammary gland. This is altogether surprising as PR-B expression and function are essential for pubertal and pregnancy associated mammary gland development in mice. Interestingly, as shown in **Chapter 4**, a recently identified main downstream mediator of PR-B function in murine mammary gland, RANKL, is still potently induced by progesterone in canine mammary tissue, which could explain the normal progesterone-dependent mammary gland development in dogs. This however also suggests that dogs have evolved an alternative compensatory mechanism for PR-B-dependent RANKL induction.

Prolonged exposure to progesterone (such as during the luteal phase) was shown to induce mammary growth hormone (GH) expression. Expression of mammary GH is not restricted to dogs, but only in this species the levels of progestin-induced mammary GH expression were reported to get so high to have endocrine effects and even cause signs of acromegaly[5,6]. We propose that the high progesterone-induced mammary GH expression in dogs might serve as a compensatory mechanism for the loss of active PR-B. Namely, progesterone-induced RANKL expression has recently been shown to be dependent on PR's tethering to Stat5[7]. As both PR isoforms are able to tether to Stat5, high mammary GH signaling might serve to increase PR-A's ability to regulate RANKL expression in absence of PR-B. Further studies will have to test this notion as well as to address the regulation of canine mammary GH expression and signaling. An intriguing hypothesis is that mammary GH expression is stimulated by PR-A and directly or indirectly inhibited by PR-B signaling. This would be in concordance with previously published observations showing that : 1) mammary GH expression in mice peaks during PR-A dominated phase[8]; 2) GH expression in dogs is associated with proliferative morphology, while PR-B stimulates alveologenesis in mice[9,10]; 3) in humans mammary GH expression increases during progression to malignancy that is often associated with disturbed PR-A to PR-B ratio[11,12]. In order to test this hypothesis it would be interesting to analyze GH expression in PRAKO and PRBKO mammary glands before and after progesterone treatment. Interestingly, in **Chapter 2** we also reported that PR-A transcript is expressed as two protein isoforms (82- and 94-kDa) that both undergo ligand-induced phosphorylation and down-regulation, implicating functional proteins. Two PR-A protein bands have been reported

previously as well[13,14], but their structural and functional aspects have never been addressed. In would therefore be of great interest to fully characterize the two PR-A protein bands especially in relation to putative regulation of GH expression in the mammary gland.

Additional aspect that remained unexplored in this thesis regards the ancestral function of PR-B. As addressed previously, PR-B function has been linked mainly to mammary gland development and function. However, multiple species alignment of available vertebrate PR sequences implies strong conservation of AF3 domain motifs (and thereby PR-B function), not only in mammals, but in all land vertebrates (data not shown). It is therefore expected that the ancestral function of PR-B extends beyond mammary gland and may for example be related to changes in reproductive biology during transition from aqueous environment. Knocking out PR-B in amphibian or reptile species may be informative in this regard.

Tumor promoting effects of progesterone signaling imply that antiprogestins might serve as therapeutic agents for breast cancer. The complexity arises from the fact that some antiprogestins have PR isoform specific functions. As such, in cultured cells, mifepristone (RU486) was shown to inhibit PR-A signaling, but in the same cells it can act as an agonist of PR-B signaling[15]. Similarly, in human breast cancer xenograft models, RU486 inhibited the growth of PR-A-overexpressing tumors and stimulated PR-B overexpressing xenografts[16]. Based on our findings regarding the low transcriptional activity of canine PR-B, RU486 could be an interesting therapeutic candidate for PR-positive canine mammary tumors, although its strong antiglucocorticoid activity may reduce its usefulness.

In addition to GH, progesterone was shown to induce canonical Wnt signaling in the benign mammary gland. Both of these signaling pathways as well as progesterone signaling often remain active during tumor progression as evidenced by the presence of PR and GH expression and overstabilization of β-catenin. There is however a subset of mammary tumors that lack PR expression but still show mammary GH expression or signs of canonical Wnt signaling, suggesting loss of progesterone control[17,18]. In **Chapter 5** we explored the possibility that in tumors lacking PR, canonical Wnt and GH signaling directly crossregulate each other's activity. For this purpose GH-responsive canine mammary tumor cell line was generated by stable

transfection with DOX-inducible GH receptor (GHR). In the respective cell line, activation of GH signaling had, however, no effect on TCF-reporter activity or endogenous Axin2 expression. Similarly, stimulation of canonical Wnt activity did not affect mammary GH or GHR expression, suggesting that the two signaling pathways are regulated by progesterone independently of each other and show no direct crossregulation. Further studies will, however, need to address the possibility of synergism between GH and canonical Wnt in regulation of other signaling pathways during tumor initiation and progression.

In benign mammary tissue progesterone was shown to induce canonical Wnt activity, presumably through upregulation of Wnt ligands[19,20]. In breast cancer, overstabilization of β-catenin is often observed but the mutations present in other types of tumors (e.g. APC and β-catenin) are rarely detected. Therefore, alternative mechanisms of canonical Wnt activation in breast cancer have been suggested, including mutations in other components of the pathway, overexpression of Wnt ligands, downregulation of antagonists and/or crossregulation by other pathways. In **Chapter 6**, we quantitatively addressed the level and the mechanism of canonical Wnt activation in canine mammary cell lines established from spontaneous primary tumors and their metastases. Using TCF-reporter assay we showed that activation of canonical Wnt signaling is frequent among canine mammary tumor cell lines. Moreover, none of the tested cell lines expressed functional PR, implicating that the activation is independent of progesterone signaling. In addition, we defined three groups of cell lines based on the level of canonical Wnt activity (low, moderate and high). Cell lines with moderate activity were all Wnt ligand dependent as their canonical Wnt activity could effectively be inhibited by treatment with the inhibitor of Wnt ligand palmitoylation and secretion (IWP-2). In contrast, cell lines with high canonical Wnt activity showed no ligand dependency and carried no mutations in the pathway members frequently mutated in other types of tumors (APC, β-catenin, GSK3β, CK1α and Axin1). Instead, we showed that the mechanism of the canonical Wnt activation in these cell lines involves overexpression of TCF-family member, LEF1. Knock down of LEF1 in these cell lines using siRNA significantly lowered TCF-reporter activity, but did not fully inhibit it. Although this might by an artifact of transient transfections it may also imply a role for additional factors. The future efforts will need to focus on deciphering mutations underlying LEF1 overexpression as well as on assessing its frequency and relevance in primary mammary tumors.

In relation to that, in **Chapter 6** we also pointed out the need for a reliable marker of canonical Wnt activity in tissue samples. Currently, two most often used markers are β-catenin overstabilization and/or nuclear localization and the expression of a target gene Axin2. Our data points out, however, that neither of these two markers is reliable enough. Namely, in canine mammary cell lines with high ligand-independent canonical Wnt activity no marked overstabilization of β-catenin was observed. Similarly, although Axin2 expression indeed potently responds to canonical Wnt pathway modulations (**Chapter 5** and **6**) in **Chapter 6** we showed that the basal expression of Axin2 in canine mammary tumor cell lines does not correlate with TCF-reporter activity. Basal Axin2 mRNA or protein levels can therefore not be used as a quantitative marker of canonical Wnt activity.

A further critical note includes the use of current canine mammary cell line models, as they appear not to be representative of primary mammary tumors. As such, none of the tested canine mammary tumor cell lines expressed functional PR, ER, GH or GHR protein, while most of the tumor specimens do. This artifact of the system may be caused by epigenetic changes due to culturing conditions resulting in a loss of expression. Alternatively, it may imply a selection of a specific (non-representative) cell type. Cell lines used in our studies were predominantly triple (ER, PR and HER2) negative or HER2 positive, but the information about the status of the primary tumors was not always available. Therefore, in order to fully explore progestin-induced signaling mechanisms in the mammary gland, there is a need for a more representative culture model such as an organoid system that would enable simultaneous culture of multiple (differentiated) cell types from both normal and tumorous tissue. In concordance, progesterone-dependent induction of RANKL expression was shown to fail in 2D cultures (such as cell lines), but was clear in intact breast tissue microstructures and a 3D organoid culture model[2,21].

To summarize, this work has addressed some of the putative mechanisms underlying progestin-induced mammary tumorigenesis in dogs. Our results support the notion that there are a lot of similarities between dogs, humans and rodents with respect to progestin-induced signaling mechanisms during mammary tumorigenesis. However, the unique aspects of progesterone-signaling in dogs make it an even more intriguing model for both fundamental and translational research.

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Nederlandse samenvatting

Borstkanker is de meest voorkomende vorm van maligniteit in vrouwen, waarvoor hormonen uit eierstokken geïdentificeerd zijn als een belangrijke risico factor. Vooral progesteron en de onderliggende signaalpaden lijken een belangrijke rol te spelen tijdens het proces van melkklier tumorigenese[1,2]. Een belangrijk aspect hierbij is dat progesteron signalering een rol kan spelen zowel tijdens de initiatie als bij de progressie van de tumor. Het is daardoor heel belangrijk om te kiezen voor een geschikt model systeem waar zowel de initiatie als de progressie fase bestudeerd kunnen worden. Honden zijn in dat opzicht een geschikt model vanwege een heel hoge progesteron-afhankelijke incidentie van melkkliertumoren. Bovendien is er een grote beschikbaarheid van honden melkklier cellijnen en het primair melkklierweefsel kan makkelijk verkregen worden van patiënten uit dierenartspraktijken. Toch zijn de mechanismen achter progesteron-geïnduceerde melkklier tumorigenese voor een groot deel niet onderzocht gebleven en er zijn geen studies geweest naar progesteron signalering in honden sinds het kloneren van de honden progesteron receptor (PR) sequentie[3].

In dit proefschrift hebben we ons eerst gericht op de rol van progesteron in honden melkklier tumor initiatie door de functie en de expressie van honden PR te bestuderen. PR komt tot expressie als twee hoofd eiwit isovormen, PR-B en het getrunceerde PR-A eiwit. In **Hoofdstuk 2** beschrijven we een intrigerende bevinding over de functie van de honden PR-B isoform. In tegenstelling tot andere zoogdieren, hebben honden meerdere mutaties binnen het AF3 domein dat essentieel is voor de transcriptionele activiteit van PR-B. Als gevolg hiervan heeft honden PR-B een zeer beperkte transcriptionele activiteit, wat blijkt uit de lage transactivatie van progesteron receptor gevoelige luciferase reporters en endogene target genen. Deze resultaten zijn bevestigd en nog duidelijker geworden in **Hoofdstuk 3**, waar we microarray expressie profielen hebben gemaakt van honden melkkliercellijnen die induceerbare humane PR-B, honden PR-B of een chimeer van honden PR-A en humane AF3 domain tot expressie bracht. In tegenstelling tot humane PR-B of chimeer, reguleerde honden PR-B slechts een hele kleine fractie van genen en in zeer beperkte mate wat ook zijn biologische relevatie ter discussie stelt. In **Hoofdstuk 2** wordt verder beschreven dat het fenomeen van lage AF3 domein-afhankelijke transcriptionele activiteit uniek lijkt voor caniden (wolf- en vos-achtigen) en correleert sterk met hun opvallende progesteron-verwante reproductie

biologie. Deze houdt in dat de langdurige luteale fase van oestrus cyclus die vaak samengaat met pseudograviditeit en in bepaalde soorten zelfs alloparentie (waar niet-reproductieve vrouwtjes kunnen lacteren en zorgen voor de nakomelingen van reproductieve vrouwtjes)[4]. Daarnaast hebben we, zoals beschreven in **Hoofdstuk 4**, een honden PR-B specifiek antilichaam gegenereerd en laten zien dat de expressie van PR-B heel laag is of zelfs afwezig in de melkklier. Dit is heel verrassend aangezien de expressie en functie van PR-B essentieel is bevonden voor de puberale en zwangerschap geassocieerde melkklier ontwikkeling in muizen. Belangwekkend, vonden we in **Hoofdstuk 4** dat de recentelijk geïdentificeerd onderliggende hoofd mediator van PR-B functie in de muizen melkklier, RANKL, is toch sterk geïnduceerd door progesteron in de honden melkklier. Dit suggereert dat honden een alternatief mechanisme hebben ontwikkeld voor de PR-B afhankelijke RANKL inductie.

Het is bekend dat de langdurige blootstelling aan progesteron (zoals tijdens de luteale fase) de expressie van groeihormoon (GH) kan stimuleren in de melkklier. Expressie van melkklier GH is niet beperkt tot honden, maar alleen in honden is het bekend dat het niveau van progesteron-geïnduceerde melkklier GH zo hoog kan worden dat het terug te vinden is in het bloed om endocriene effecten en zelfs acromegalie kan veroorzaken[5,6]. We stellen voor dat de sterke progesteron-geïnduceerde melkklier GH expressie in honden dient als een compensatie mechanisme voor het verlies van actief PR-B. Progesteron-afhankelijke inductie van RANKL is recentelijk aangetoond afhankelijk te zijn van binding van PR aan Stat5[7]. Aangezien beide PR isovormen kunnen binden aan Stat5, zou de hoge GH concentratie in de melkklier PR-A kunnen helpen bij de regulatie van RANKL in de afwezigheid van PR-B. Toekomstige studies zijn nodig om hier het antwoord op te geven evenals op andere vragen rond de regulatie van honden melkklier GH expressie en signalering. Een intrigerende hypothese is dat de expressie van melkklier GH is gestimuleerd door PR-A en direct of indirect geremd door PR-B signalering. Dit komt overeen met de gepubliceerde bevindingen dat: 1) melkklier GH expressie in muizen een piek bereikt tijdens PR-A gedomineerde fase[8]; 2) GH expressie in honden geassocieerd is met proliferatieve morfologie, terwijl PR-B juist de vorming van alveoli stimuleert in muizen[9,10]; 3) in mensen neemt GH expressie toe tijdens de progressie naar hogere maligniteit, die geassocieerd is met veranderingen in de verhouding tussen PR-A en PR-B[11,12]. Om deze hypothese te testen

zou het interessant zijn om de expressie van GH te analyseren in PRAKO en PRBKO melkklieren voor en na de blootstelling aan progesteron. Zoals beschreven in **Hoofdstuk 2** is het ook vermeldingswaard dat het PR-A transcript tot expressie komt in twee eiwit isovormen (82- en 94-kDa) die allebei een ligand-afhankelijke fosforyleringen en afbraak ondergaan, wat suggereert dat ze functioneel zijn. Twee eiwit isovormen van PR-A zijn al eerder opgemerkt[13,14], maar er is nog niets bekend over hun structurele en functionele aspecten. Het zou daardoor zeer interessant zijn om de twee PR-A isovormen volledig te karakteriseren vooral in relatie tot de mogelijke regulatie van melkklier GH expressie.

Een ander aspect dat verder onbeschreven is gebleven in dit proefschrift betreft de functie van PR-B in evolutionaire voorouders. Zoals eerder vermeld is de functie van PR-B voornamelijk verbonden met de melkklierontwikkeling en functie. Vergelijken van alle beschikbare PR sequenties van gewervelden dieren impliceert echter een sterk behoud van AF3 domein motieven (en dus ook PR-B functie), niet alleen in zoogdieren, maar in alle land gewervelde soorten. Het is daarom te verwachten dat de oorspronkelijke functie van PR-B buiten de melkklier ligt en mogelijk verbonden is met veranderingen in reproductie biologie gedurende de overgang uit waterige omgeving. Een knock-out van PR-B in een amfibie of een reptiel zou informatief kunnen zijn in dit aspect.

Tumor stimulerende effecten van progesteron signalering suggereert dat antiprogestagenen mogelijk als therapeutische middelen kunnen gebruikt worden voor het remmen van borstkanker. Het lastige is wel dat sommige antiprogestagenen een PR isovorm specifieke werking hebben. Het is bijvoorbeeld bekend dat mifepriston (RU486) in gekweekte cellen de PR-A signalering remt, maar in dezelfde cellen kan werken als agonist van PR-B[15]. Evenzo, in een humaan borstkanker xenograft model, remde RU486 de groei van PR-A over-expresserende tumoren en stimuleerde de groei in PR-B over-expresserende xenografts[16]. Gebaseerd op onze bevinding over de lage transcriptionele activiteit van honden PR-B zou RU486 een interessante therapeutische kandidaat kunnen zijn voor PR-positieve honden melkkliertumoren, ondanks dat zijn sterke anti-glucocorticoïde werking zijn bruikbaarheid kan verlagen.

Het is bekend dat progesteron naast GH, ook de canonieke Wnt signalering in de benigne melkklier kan stimuleren. Beide signaalpaden, net als progesteron signalering, blijven vaak actief gedurende tumor progressie wat te merken is aan de aanwezigheid van PR en GH expressie en verhoogde stabilisatie van β-catenine. Er is echter een deel van de melkklier tumoren zonder PR expressie die nog wel GH expressie kennen en canonieke Wnt activiteit vertonen wat een verlies van controle door progesteron suggereert [17,18]. In **Hoofdstuk 5** is onderzocht of in de PR negatieve tumoren canonieke Wnt en GH elkaars activiteit direct kunnen reguleren. Hiertoe is een GH-responsieve honden melkklier tumor cellijn gegenereerd door een stabiele transfectie met DOX-induceerbare GH receptor (GHR). In deze cellijn had activatie van GH signalering geen effect op de activiteit van een TCF-reporter noch op endogene expressie van Axine2. Vergelijkbaar had de activatie van canonieke Wnt signalering geen effect op endogeen GH of GHR expressie, suggererend dat de twee signaal paden onafhankelijk van elkaar zijn gereguleerd door progesteron en geen directe crossregulatie vertonen. Toch zijn er verdere studies nodig om uit te zoeken of er mogelijk een synergisme bestaat tussen GH en canonieke Wnt signalering gedurende tumor initiatie en progressie.

In het benigne melkklier weefsel stimuleert progesteron canonieke Wnt activiteit waarschijnlijk via verhoging van de expressie van Wnt liganden[19,20]. In borstkanker wordt verhoogde stabiliteit van β-catenine vaak gezien, maar de mutaties aanwezig in andere type tumoren (e.g. APC en β-catenine) worden zelden gedetecteerd. Er zijn daarom alternatieve mechanismen voorgesteld voor de activatie van canonieke Wnt signalering in borstkanker zoals mutaties in anderen componenten van het signaal pad, verhoogde expressie van Wnt liganden, verlaagde expressie van antagonisten en/of crossregulatie met andere signaalpaden. In **Hoofdstuk 6** is de activiteit van canonieke Wnt signalering kwantitatief onderzocht in honden melkklier cellijnen gegenereerd uit primaire tumoren en hun metastasen. Door middel van TCF-reporter assay lieten we zien dat de activatie van canonieke Wnt signalering vaak voorkomend is in honden melkklier tumor cellijnen. Omdat geen van de geteste cellijnen functioneel PR tot expressie bracht, suggereert dit dat de activiteit van canonieke Wnt progesteron onafhankelijk is. Daarnaast, konden de cellijnen ingedeeld worden in drie verschillende groepen op basis van hun canonieke Wnt activiteit (laag, middelmatig en hoog). Cellijnen met middelmatige activiteit waren allemaal Wnt ligand

afhankelijk omdat hun activiteit volledig te remmen was door behandeling met de inhibitor van Wnt ligand palmitoylering en secretie (IWP-2). Daarentegen, cellijnen met hoge canonieke Wnt activiteit waren ligand onafhankelijk en hadden geen mutaties in de leden van het signaalpad die vaak gemuteerd zijn in andere type tumoren (APC, β-catenine, GSK3β, CK1α en Axine1). In plaats daarvan, laten we zien dat het mechanisme van canonieke Wnt activatie in deze cellijnen te maken heeft met de verhoogde expressie van TCF-familielid, LEF1. 'Knock-down' van LEF1 expressie in deze cellijnen door middel van siRNA resulteerde in een significante daling van TCF-reporter activiteit, maar kon het niet volledig afremmen. Dit zou een artefact kunnen zijn van een transiente transfectie, maar kan ook duiden op betrokkenheid van andere factoren. Verder onderzoek zou zich moeten richten op het ontcijferen van de mutaties oorzakelijk voor de verhoogde expressie van LEF1, evenals om zijn frequentie en relevantie in de primaire tumoren te evalueren. In verband hiermee wijzen we in **Hoofdstuk 6** op de noodzaak voor nieuwe betrouwbare reporters van canonieke Wnt activiteit in weefsels. Tegenwoordig zijn de twee meest gebruikte reporters verhoogde stabilisatie van β-catenine en/of zijn nucleaire localisatie, en de expressie van het target gen Axine2. Onze gegevens duiden erop dat beide reporters niet betrouwbaar genoeg zijn. In honden melkklier cellijnen met hoge canonieke Wnt activiteit is er namelijk geen sprake van verhoogde stabilisatie van β-catenine. Evenzo, ondanks dat Axine2 expressie heel gevoelig is voor de veranderingen in canonieke Wnt activiteit (**Hoofstuk 5** en **6**) laten we in **Hoofdstuk 6** zien dat de basale expressie van Axine2 in honden melkklier cellijnen niet correleert met de TCF-reporter activiteit. Basale Axine2 mRNA expressie of eiwit niveau kan daarom niet gebruikt worden als een kwantitatieve reporter van canonieke Wnt activiteit.

Een andere kritische opmerking betreft het huidige gebruik van honden cellijn modellen, omdat ze niet volledig representatief lijken voor de primaire tumoren. Alle gebruikte cellijnen waren namelijk negatief voor functionele PR, ER, GH en GHR, terwijl de meeste primaire tumoren positief zijn. Dit artefact zou kunnen zijn veroorzaakt door epigenetische veranderingen tijdens het kweken die tot het verlies van expressie hebben geleid. Alternatief, impliceert dit selectie van een specifiek (niet-representatief) celtype. Cellijnen die gebruikt zijn in deze studie waren voornamelijk tripel (ER, PR en HER2) negatief of HER2 positief, maar de status van de

primaire tumor was niet altijd beschikbaar. Om progesteron-geïnduceerde signalering paden in de melkklier volledig te kunnen onderzoeken is daarom een representatiever kweekmodel nodig, zoals het organoïden systeem, waar het mogelijk is om tegelijkertijd meerdere gedifferentieerde celtypen te kweken uit zowel normaal als tumor weefsel. Dit blijkt ook uit het feit dat progesteron-afhankelijke inductie van RANKL expressie niet lukt in een 2D kweeksysteem (zoals cellijnen), maar heel duidelijk is in intacte melkklier microstructuren en in een 3D organoid kweekmodel[2,21].

Samenvattend, in dit proefschrift hebben we gekeken naar de progesteron-geïnduceerde signaleringsmechanismen tijdens melkklier tumorigenese bij honden. Onze resultaten bevestigen dat er tussen hond, mens en knaagdieren veel gemeenschappelijke aspecten zijn met betrekking tot progesteron-geïnduceerde signaalpaden tijdens melkklier tumorigenese. Echter, de unieke aspecten van progesteron signalering in honden maken het zelfs een interessanter model voor zowel fundamenteel als translationeel onderzoek.

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List of abbreviations

?RE	Other/additional response element
AF	Activation function domain
APC	Adenomatous polyposis coli
BSA	Bovine serum albumin
BUS	Progesterone receptor isoform B-specific upstream segment
cAF3	Canine activation function 3 domain
CDK2	Cyclin dependent kinase 2
cGHR	Canine growth hormone receptor
CHO-K1	Chinese hamster ovary cell line
CK1	Casein kinase 1
CNMm-6TR	Canine mammary cell line CNMm stably transfected with Tet-R construct
CNMm-cGHR	Canine mammary cell line CNMm stably transfected with canine growth hormone receptor
cPR	Canine progesterone receptor
cPR-A	Canine progesterone receptor isoform A
cPR-B	Canine progesterone receptor isoform B
Ct	Cycle threshold
DBD	DNA binding domain
DCIS	Ductal carcinoma in situ
Dkk	Dikkopf
DMSO	Dimethylsulfoxide
DOX	Doxycycline
E	Estrogen
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Estrogen receptor
ERα	Estrogen receptor isoform alpha
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
FKBP5	FK506 binding protein 5
FOP	pFOPFLASH luciferase construct containing mutated TCF binding sites
Fz or Frz	Frizzled
GH	Growth hormone
GHR	Growth hormone receptor
GnRH	Gonadotropin-releasing hormone

GSK-3β	Glycogens synthase kinase-3 beta
H or HR	Hinge region
hAF3-cPR	Canine progesterone receptor isoform A coupled to the human activation function 3 domain
hBUS	Human progesterone receptor isoform B-specific upstream segment
hPR-A	Human progesterone receptor isoform A
hPR-Ala	Human progesterone receptor with canine-like polyalanine insert
hPR-B	Human progesterone receptor isoform B
HEK-rGHR	Human embryonic kidney cell line stably transfected with rabbit growth hormone receptor
HER2	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
IDC	Infiltrating ductal carcinoma
IF	Inhibition function domain
IGF-1	Insulin-like growth factor 1
IL-8	Interleukin 8
JAK2	Janus kinase 2
JNK	c-Jun N-terminal protein kinase
kDa	Kilo Dalton (molecular mass unit)
KO	Knockout
LBD	Ligand binding domain
LCIS	Lobular carcinoma in situ
LEF1	Lymphoid enhancer-binding factor 1
LGR	Leucin-rich repeat containing G protein-coupled receptor
LH	Luteinizing hormone
LiCl	Lithium chloride
LRP5/6	LDL-related protein 5 and 6
MAPK	Mitogen-activated protein kinase
miRNA	Micro RNA
MMTV	Mouse mammary tumor virus
MMTV-luc	Luciferase construct driven by the mouse mammary tumor virus promoter with a palindromic progesterone response element
MPA	Medroxyprogesterone acetate (progestin)
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MT1	Metallothionein 1
NLS	Nuclear localization signal
P or P4	Progesterone

PCR	Polymerase chain reaction
PGRMC1	Progesterone receptor membrane component 1
PGRMC2	Progesterone receptor membrane component 2
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PR or PGR	Progesterone receptor
PR-A	Progesterone receptor isoform A
PRAKO	Progesterone receptor isoform A knockout
PR-B	Progesterone receptor isoform B
PRBKO	Progesterone receptor isoform B knockout
PRE	Progesterone response element
PRE2-luc	Luciferase construct driven by tyrosine aminotransferase promoter with two tandem palindromic progesterone response elements
PRL	Prolactin
PTEN	Phosphatase and tensin homolog
qPCR or qRT-PCR	Quantitative (real-time) polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa B ligand
rGHR	Rabbit growth hormone receptor
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay buffer
Rps19	Ribosomal protein S19
Rspo	R-spondin
RT	Room temperature
RU486	Mifepristone (antiprogestin)
RU534	Aglepristone (antiprogestin)
S	Phosphorylated serine residue
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m.	Standard error of the mean
SERM	Selective estrogen receptor modulators
sFRP1	Secreted frizzled-related protein 1
SGK	Serum and glucocorticoid regulated kinase
SNP	Single nucleotide polymorphism
Spi2.1	Serum protease inhibitor 2.1
Spi-luc	Luciferase construct driven by the promoter of serum protease inhibitor 2.1 gene
Src or c-Src	Proto-oncogene tyrosine-protein kinase (cellular) Src
Stat	Signal transducer and activator of transcription

SUMO	Small ubiquitin-like modulator
T	Phosphorylated threonine residue
T47D-Y	PR negative subclone of a human cell lines isolated from a metastasized mammary tumor
TBS	Tris-buffered saline
TCF	T-cell factor
TEB	Terminal end bud
Tet-free	Tetracylcline free
TOP	pTOPFLASH luciferase construct containing functional TCF binding sites
UTR	Untranslated region
ve	Empty vector
VEGF	Vascular epidermal growth factor
Wt	Wild type

Curriculum Vitae

Ana Gracanin was born on 5th of June 1983 in Zenica, Bosnia and Hercegovina. She attendend XV. Gymnasium in Zagreb, Croatia. In 2002 she obtained propaedeutic diploma in Biology from Wageningen University, The Netherlands. She continued her education at the Utrecht University, The Netherlands were she obtained *cum laude* BSc degree in Biology in 2004. Subsequently, she enrolled in and, in 2008, successfully completed two Master programs: Animal Biology and Bioveterinary Sciences (*cum laude*). She was awarded a Mosaic grant from the Dutch organization for scientific research (NWO) that enabled her to start her PhD project at the Department of Clinical Sciences for Companion Animals at the Utrecht University in 2008. The results of this project are presented in this thesis and will be publicly defended on 8th of September 2015. In 2012, she became a research technician in the group of Prof. Hans Clevers, Hubrecht Institute, The Netherlands. From 2014, she is in training to become a scientist in the HUB foundation for Organoid Technology, The Netherlands.

List of Publications

Boj SF, Hwang CI, Baker LA, Chio II, Engle DD, Corbo V, Jager M, Ponz-Sarvise M, Tiriac H, Spector MS, **Gracanin A**, Oni T, Yu KH, van Boxtel R, Huch M, Rivera KD, Wilson JP, Feigin ME, Öhlund D, Handly-Santana A, Ardito-Abraham CM, Ludwig M, Elyada E, Alagesan B, Biffi G, Yordanov GN, Delcuze B, Creighton B, Wright K, Park Y, Morsink FH, Molenaar IQ, Borel Rinkes IH, Cuppen E, Hao Y, Jin Y, Nijman IJ, Iacobuzio-Donahue C, Leach SD, Pappin DJ, Hammell M, Klimstra DS, Basturk O, Hruban RH, Offerhaus GJ, Vries RG, Clevers H, Tuveson DA. Organoid models of human and mouse ductal pancreatic cancer. *Cell.* 2015 Jan 15;160 (1-2):324-38.

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