Metalloproteinase axes increase β-catenin signaling in primary mouse mammary epithelial cells lacking TIMP3

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Accepted 17 January 2007
Journal of Cell Science 120, 1050-1060 Published by The Company of Biologists 2007
doi:10.1242/jcs.003335

Summary

Multiple cancers exhibit mutations in β-catenin that lead to increased stability, altered localization or amplified activity. β-catenin is situated at the junction between the cadherin-mediated cell adhesion and Wnt signaling pathways, and TIMP3 functions to alter β-catenin signaling. Here we demonstrate that primary mouse embryonic fibroblasts (MEFs) and mammary epithelial cells (MECs) deficient in *Timp3* have increased β-catenin signaling. Functionally, the loss of TIMP3 exerted cell-type-specific effects, with *Timp3*$^{-/-}$ MEFs being more sensitive and *Timp3*$^{-/-}$ MECs more resistant to EGTA-induced cell detachment than the wild type. *Timp3*$^{-/-}$ MECs had higher dephosphorylated β-catenin levels and increased β-catenin transcriptional activity as measured by TCF/LEF-responsive reporter assays. Real-time PCR analysis of β-catenin target genes in MEFs and MECs showed no alteration in *Myc*, decreased *Ccnd1* (cyclin D1) and increased *Mmp7* mRNA levels upon loss of TIMP3, with the latter occurring only in epithelial cells. Recombinant TIMP3 and synthetic metalloproteinase inhibitors reverted the increase in dephosphorylated β-catenin, decrease in *Ccnd1* gene expression and increase in *Mmp7* gene expression. Physiologically, *Timp3*$^{-/-}$ mammary glands displayed accelerated mammary ductal elongation during pubertal morphogenesis. Gain-of-function studies using slow-release TIMP-containing pellets revealed distinct effects of individual TIMPs on ductal morphogenesis. Recombinant TIMP1, TIMP3 and TIMP4 inhibited ductal elongation whereas TIMP2 promoted this process.

Key words: TIMP3, β-catenin, Mammary epithelial cells, MMP7, Cyclin D1

Introduction

The gene encoding β-catenin is commonly mutated in cancers of the colon and connective tissues and the protein is upregulated in a significant proportion of breast cancers (Cheon et al., 2002; Lin et al., 2000; Moon et al., 2004; Nelson and Nusse, 2004). β-catenin–activating mutations lead to increased β-catenin stability and transcriptional activity. Indeed, the potency of β-catenin as a protooncogene is linked to its functioning in multiple cellular compartments including the plasma membrane, cytoplasm and nucleus. Two main pathways impinge on the function of β-catenin: the cadherin-mediated cell-cell adherens junctions and the canonical Wnt signaling pathway (Nelson and Nusse, 2004). Upon a Wnt signal, GSK3β can no longer phosphorylate and tag casein kinase-primed β-catenin for ubiquitin-mediated degradation. β-catenin then translocates into the nucleus and activates the transcription of many genes, several of which are implicated in cancer progression. In addition, β-catenin can bind to the cytoplasmic tail of cadherins and thereby participate in cell-cell adhesion. Thus, β-catenin sits at the junction between cell-cell adhesion and the Wnt pathway (Hatsell et al., 2003).

Matrix metalloproteinases (MMPs) are extracellular proteases that are inhibited by a family of endogenous inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) (Lambert et al., 2004; Puente et al., 2003). The MMP/TIMP proteolytic axis regulates the turnover of extracellular matrix (ECM) proteins, and it was through this function that MMPs and TIMPs were identified for their role in tumor cell invasion and metastasis. Numerous studies have since expanded the range of MMP substrates to include cell adhesion molecules, cytokines, growth factors, growth factor binding proteins and receptors (George and Dwivedi, 2004; Hojilla et al., 2003; Mohammed et al., 2003; Vu and Werb, 2000). By the proteolytic cleavage of such diverse substrates, MMPs and TIMPs influence the extracellular microenvironment and alter cell fate (Egeblad and Werb, 2002; Hojilla et al., 2003).

We have shown previously that MMP/TIMP proteolysis affects cadherin stability. The downregulation of TIMP1 transformed the otherwise normal fibroblasts to become tumorigenic in mice (Khokha et al., 1989). These cells exhibited destabilized cell-cell contact because of decreased cadherin levels and the decreased recruitment of β-catenin to the cell membrane (Ho et al., 2001). MMP3 overexpression in mammary epithelial cells (MECs) is associated with diminished E-cadherin and β-catenin levels (Lochter et al., 1997; Sternlicht et al., 1999). MMP3 and MMP7 promote invasiveness of MDCK and MCF-7 tumor lines by releasing the E-cadherin ectodomain and reducing adhesion; an effect inhibited by TIMP2 (Noe et al., 2001). These latter studies have implicated MMP-mediated E-cadherin shedding as a mechanism for dissemination of cancer cells through a process

known as epithelial-to-mesenchymal transition (Thiery, 2002). A similar role for MMP7 is suggested in a prostate cancer cell line in response to the scatter factor, hepatocyte growth factor (Davies et al., 2001). This highlights the role of MMPs and TIMPs in influencing cadherin and/or β-catenin stability and cell-cell adhesion (McGuire et al., 2003; Reiss et al., 2005).

Among the four TIMPs, TIMP3 has distinctive characteristics. A TIMP3 mutation leads to Sorsby's fundus dystrophy, a retinal degenerative disease (Weber et al., 1994). Although other TIMPs are soluble upon secretion from the cell, TIMP3 remains bound to the ECM (Leco et al., 1994; Pavloff et al., 1992) through sulfated proteoglycans moieties (Yu et al., 2000). Evidence exists that either very high TIMP3 expression or the loss of TIMP3 promote apoptosis (Ahonen et al., 2003; Bond et al., 2002; Fata et al., 2001; Mohammed et al., 2004). Finally, TIMP3 also inhibits several members of the ADAM (a disintegrin and a metalloproteinase) protease family, such as ADAM10 (Amour et al., 2000), ADAM17 (Black et al., 1997; Lee et al., 2001; Mohammed et al., 2004), ADAM12S (Loechel et al., 2000) and ADAM-TSs (Kashiwagi et al., 2001). TIMP3 inhibition of ADAM17 is crucial for controlling TNF-mediated inflammation (Mohammed et al., 2004). In this study, we investigate the role of TIMP3 on cell-cell adhesion, β-catenin subcellular localization and transcriptional activity in primary cells. Our work shows that loss of TIMP3 alters cell-cell contact in a cell-type-specific manner. β-catenin signaling is increased and expression of β-catenin target genes is altered in an MMP- and ADAM-regulated manner upon TIMP3 deficiency in mammary epithelial cells. We also identify distinct effects of TIMPs on mammary ductal elongation during virgin morphogenesis.

Results

Cadherin-independent impairment in cell-cell contact of *Timp3*$^{-/-}$ MEFs

We previously showed that the downregulation or loss of TIMP1 in fibroblasts leads to destabilization of cell-cell adhesion (Ho et al., 2001). Using primary MEFs in the present study, we compared the ability of wild-type (WT) and *Timp3*$^{-/-}$ MEFs to form aggregates, as a measure of the quality of their cell-cell contact. Although WT MEFs readily formed aggregates as early as 15 minutes and large aggregates by 45 minutes, only a few small *Timp3*$^{-/-}$ MEF aggregates were seen after 45 minutes (Fig. 1A). Ca^{2+} chelation by EGTA treatment in tissue culture systems provides another measure of cell-cell adhesion. This resulted in earlier lifting of *Timp3*$^{-/-}$ fibroblast monolayers compared with WT (Fig. 1B,C). These observations suggested altered cadherin function between WT and *Timp3*$^{-/-}$ MEFs because cadherin function is Ca^{2+} dependent and required for cell-cell aggregation (Rothen-Rutishauser et al., 2002). However, the levels of total and each of the specific cadherins we assessed including epithelial (E-) and mesenchymal (N-, H- and OB-) cadherins, were the same in WT and *Timp3*$^{-/-}$ MEFs (Fig. 1D). Using subcellular fractionation, western blot analysis and confocal microscopy, we further noted no differences between levels of membrane-localized cadherins (data not shown). Densitometric analyses of the western blot panels for the three independent primary cultures of each genotype confirmed a lack of significant difference in cadherin levels.

Similar to the expression of cadherins, the total levels of β-catenin were unaltered in MEFs between genotypes (Fig. 1D). The levels of cytoplasmic and membrane-bound β-catenin, as well as the colocalization with pan-cadherin or N-cadherin, remained similar in WT and *Timp3*$^{-/-}$ MEFs as assessed by subcellular fractionation, immunofluorescence and confocal microscopy (data not shown). We further used confocal microscopy to test whether Ca^{2+} chelation differentially perturbed this colocalization in WT and *Timp3*$^{-/-}$ MEFs and found no difference (data not shown).

Timp3$^{-/-}$ MECs are resistant to EGTA-induced detachment

Although fibroblasts can form cell-cell contact (Ho et al., 2001; Reiss et al., 2005), we also used mammary epithelial cells, which are more dependent on this phenomenon. In contrast to *Timp3*$^{-/-}$ MEFs, primary MECs lacking TIMP3 were more resistant to EGTA-induced detachment (Fig. 2A). Wild-type epithelial islands had dispersed after 2 hours, whereas *Timp3*$^{-/-}$ epithelial islands only began to disperse at 4 hours. Upon EGTA treatment, cell rounding of WT MECs was observed at 1 hour giving them a distinct morphology compared with the null cells. Cell rounding occurred much later, at 6 hours in *Timp3*$^{-/-}$ MECs (data not shown). Immunofluorescence showed a similar pattern of E-cadherin and β-catenin colocalization in *Timp3*$^{-/-}$ and WT MECs during EGTA treatment, except that timing of this colocalization was delayed in *Timp3*$^{-/-}$ MECs, consistent with their delayed detachment during Ca^{2+} chelation (data not shown).

Increased nuclear β-catenin levels in *Timp3*$^{-/-}$ MECs

Pools of primary MECs were generated using all ten mammary glands of five virgin WT and five *Timp3*$^{-/-}$ female mice. WT and *Timp3*$^{-/-}$ MECs were positive for the epithelial marker cytokeratin-18 and expressed very low levels of the mesenchymal marker vimentin (Fig. 2C), confirming the epithelial cell purity of our preparations. Although pan-cadherin and P-cadherin levels were similar between WT and *Timp3*$^{-/-}$ MECs (data not shown), E-cadherin increased in the absence of TIMP3 (Fig. 2B,C). Similarly, total β-catenin levels were almost double in *Timp3*$^{-/-}$ MECs, compared with the WT cells (Fig. 2B,D). Subcellular fractionation showed increased nuclear β-catenin in the absence of TIMP3 (Fig. 2E), and immunofluorescence showed that membrane-associated β-catenin did not change in MECs with respect to the TIMP3 status (data not shown). We next examined dephosphorylated β-catenin levels. These were much higher in *Timp3*$^{-/-}$ than WT MECs in whole cell lysates and in nuclear fractions (Fig. 2F).

Wnt3A treatment promotes β-catenin recruitment to the membrane in *Timp3*$^{-/-}$ MECs

Next, we stimulated primary cell cultures with Wnt3A, which directly activates the canonical Wnt signaling pathway (Willert et al., 2003) in fibroblasts (Lee et al., 1999) and mammary epithelial cells (Haertel-Wiesmann et al., 2000). Mock stimulation with the control W0 had no effect on β-catenin levels, whereas Wnt3A treatment led to an overall increase in β-catenin in MECs of both genotypes (Fig. 3A). Wnt3A led to nuclear β-catenin accumulation with some increase in membrane-associated signal in WT MECs (Fig. 3A). A consistent qualitative observation was that the increase in membrane-bound β-catenin was more pronounced in *Timp3*$^{-/-}$

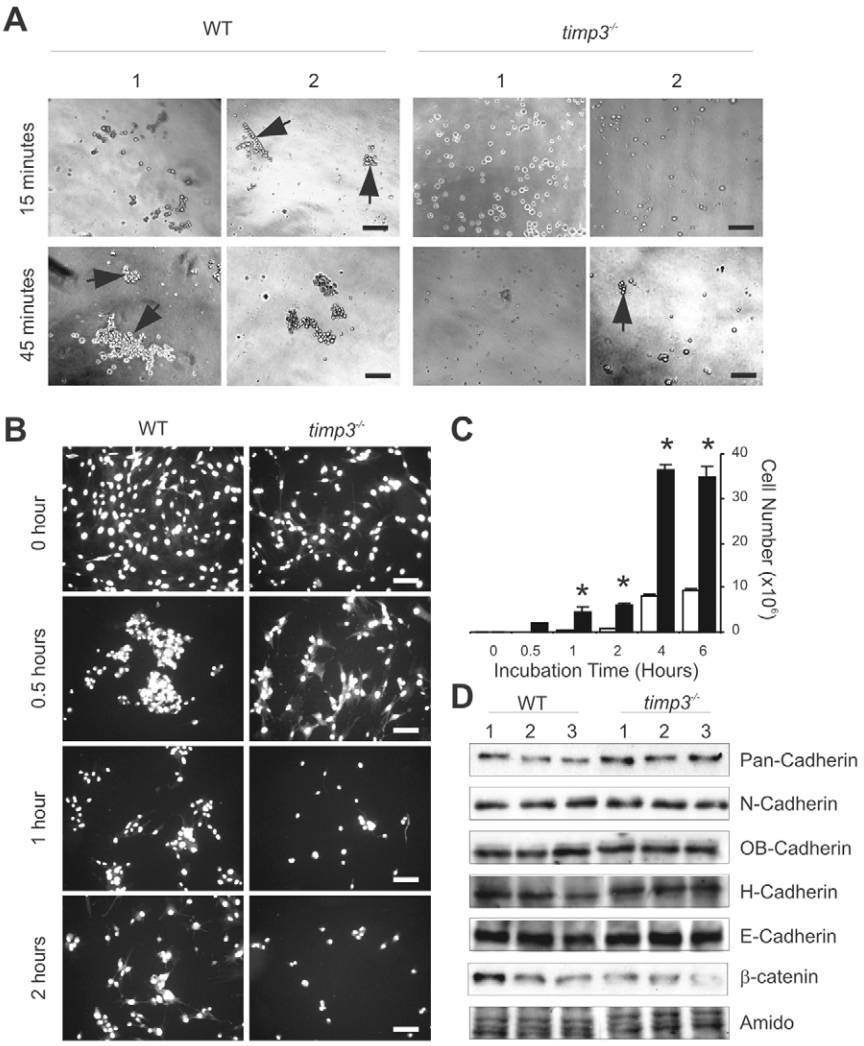

Fig. 1. Decreased cell aggregation and sensitivity to EGTA-induced detachment in primary *Timp3*$^{-/-}$ MEFs. (A) Representative examples of aggregation assay with independent MEF preparations. WT aggregates (arrows) were evident at 15 minutes and large aggregates seen at 45 minutes. *Timp3*$^{-/-}$ MEF aggregates (arrows) did not form until 45 minutes and were much smaller in size (fewer cells) than WT aggregates. Bars, 70 μm. (B) Representative images of fibroblast monolayers in a Ca^{2+} chelation assay. *Timp3*$^{-/-}$ MEFs detached before WT controls, as assessed by Hoechst staining and fluorescence microscopy of the cells remaining on the coverslips after the indicated time of EGTA treatment. Bars, 70 μm. (C) The number of viable cells in the supernatant following EGTA-induced detachment, assessed using Trypan Blue exclusion, showed higher numbers of *Timp3*$^{-/-}$ cells at all incubation times (*P<0.04; □, WT; ■, *Timp3*$^{-/-}$). Student's *t*-test was performed to assess statistical significance between WT and *Timp3*$^{-/-}$. Values are expressed as mean ± s.e.m. (D) Western blot analyses of whole cell lysates from three of the five independent clones of WT and *Timp3*$^{-/-}$ MEFs, probed with the indicated antibodies. Amido Black staining was used to assess protein loading.

MECs treated with Wnt3A, suggesting a redistribution from cytoplasmic to plasma membrane (Fig. 3A, arrowhead). Our parallel experiments in the fibroblast cultures showed that Wnt3A treatment led to nuclear β-catenin accumulation in both WT and *Timp3*$^{-/-}$ cells (Fig. 3B, arrow). These data suggest that stimulation of Wnt/β-catenin signaling in the absence of TIMP3 may lead to a differential redistribution of β-catenin in epithelial cells versus fibroblasts. Cell-type-specific effects on cell adhesion have also been described for Wnt1 (Giarre et al., 1998).

Increased β-catenin transcriptional activity and selective gene response in the absence of exogenous Wnt in *Timp3*$^{-/-}$ cells

We next determined the effect of TIMP3 loss on β-catenin-dependent transcriptional activity in epithelial cells and fibroblasts in the absence of Wnt stimulation. We transfected MEFs and MECs with a luciferase reporter driven by a β-catenin/TCF/LEF-responsive promoter and a β-galactosidase reporter that serves as a transfection control. In fibroblast cultures, the β-catenin transcriptional activity in *Timp3*$^{-/-}$ MEFs was 45% higher than in WT MEFs (Fig. 4A). This difference was far greater in MEC cultures, with β-catenin transcriptional activity being 300% higher in *Timp3*$^{-/-}$ than in WT cells (Fig. 4A). We tested whether this increased activity correlated with increased mRNA levels of β-catenin transcriptional targets, by measuring the levels of three mRNAs reportedly regulated by β-catenin: *Myc* (He et al., 1998), *Ccnd1* (cyclin D1) (Tetsu and McCormick, 1999) and *Mmp7* (Crawford et al., 1999). TaqMan RT-PCR analyses (Applied Biosystems) showed that levels of *Myc* mRNA did not change between genotypes in either cell type (data not shown). Surprisingly, the *Ccnd1* mRNA level was downregulated with TIMP3 loss in both MECs and MEFs (Fig. 4B). By contrast, the *Mmp7* mRNA level was elevated tenfold in *Timp3*$^{-/-}$ MECs, but was undetectable in fibroblasts (Fig. 4C). These data indicate that despite an increase in β-catenin transcriptional activity in the absence of TIMP3, only selected β-catenin target genes are affected. Its loss correlates with a consistent suppression of *Ccnd1* and a profound elevation of *Mmp7* gene expression, specifically in epithelial cells.

MMP- and ADAM-dependent regulation of β-catenin signaling and activity in *Timp3*$^{-/-}$ MECs

TIMP3 inhibits several MMP and specific ADAM proteases. We asked whether the increased β-catenin activity upon TIMP3 loss was related to increased proteolytic activity. MECs were treated with: (1) recombinant N-terminal TIMP3

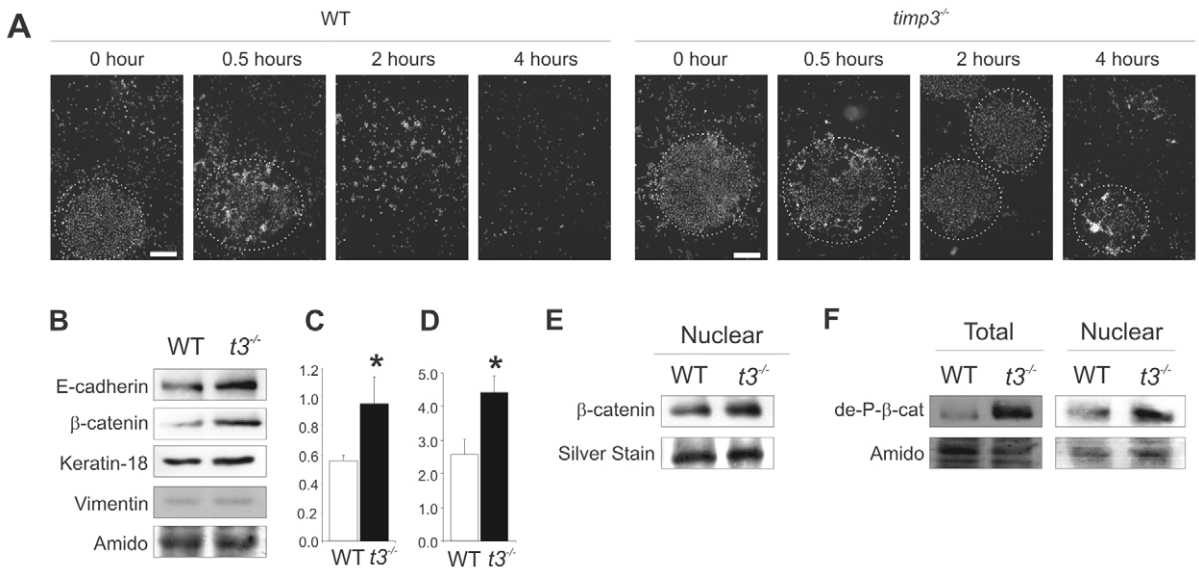

Fig. 2. Primary *Timp3*$^{-/-}$ MECs are more resistant to EGTA-induced cell detachment and have higher β-catenin levels. (A) Representative images from at least three different experiments. An increased number of *Timp3*$^{-/-}$ epithelial islands (outlined with dotted circles) remain following EGTA treatment as assessed by Hoechst staining and fluorescence microscopy. Bars, 2 mm. (B) Representative western blots of whole-cell lysates from WT and *Timp3*$^{-/-}$ (*t3*$^{-/-}$) MECs probed with the indicated antibodies. Quantification by densitometry of (C) E-cadherin signal and (D) β-catenin signal (□, WT; ■, *Timp3*$^{-/-}$; **P*<0.05). (E) Western blot analyses of nuclear lysates of WT and *Timp3*$^{-/-}$ MECs using an anti-β-catenin antibody. (F) MEC total and nuclear lysates subjected to western blotting using an anti-dephosphorylated β-catenin (de-P-β-cat). Panels B-F represent one of three pools of primary mammary epithelial cultures. Each MEC pool was harvested from all ten mammary glands of five WT and five *Timp3*$^{-/-}$ female mice. Amido Black and silver staining were used to assess protein loading. Student's *t*-test was performed to assess statistical significance between WT and *Timp3*$^{-/-}$. Bar graphs were expressed as the mean ± s.e.m.

which harbors the metalloproteinase inhibitory domain (Ahonen et al., 2003); (2) GM6001, a broad spectrum metalloproteinase inhibitor active against both MMPs and ADAMs; and (3) PD166793, an MMP-specific but ADAM-insensitive inhibitor (Chapman et al., 2003; Kassiri et al., 2005). Addition of recombinant TIMP3 lowered dephosphorylated β-catenin levels in *Timp3*$^{-/-}$ MECs to that of WT cells (Fig. 5A). Treatment with GM6001 increased the levels of dephosphorylated β-catenin in WT MECs to that seen in untreated *Timp3*$^{-/-}$ MECs, whereas PD166793 decreased this active signaling pool of β-catenin in *Timp3*$^{-/-}$ MECs (Fig. 5A). The effect of each inhibitor was tested using two different concentrations for 6 and 24 hours and the most pronounced changes in dephosphorylated β-catenin were observed at 24 hours with the higher concentration (data not shown). This indicated that the increase in dephosphorylated β-catenin in *Timp3*$^{-/-}$ MECs was influenced by metalloproteinase activity.

We then determined whether metalloproteinase-inhibitor-dependent reversal of dephosphorylated β-catenin levels subsequently altered β-catenin target genes. The repression of *Ccnd1* in *Timp3*$^{-/-}$ MECs was rescued by all three metalloproteinase inhibitors compared with the untreated *Timp3*$^{-/-}$ control (Fig. 5B). In a similar manner, the increase in *Mmp7* in untreated *Timp3*$^{-/-}$ MECs was significantly mitigated by rTIMP3, GM6001 and PD166793 treatments (Fig. 5C). We also observed a decrease in *Ccnd1* mRNA levels in WT MECs specific to PD166793 treatment. Although natural and synthetic metalloproteinase inhibitors directly rescued the specific gene responses created by TIMP3 deficiency, their effects on dephosphorylated β-catenin levels are far more complex and require further study.

Selective gene responses upon GSK3β and E-cadherin inhibition

Next, we determined the contribution of the canonical Wnt signaling pathway and E-cadherin to β-catenin activity in *Timp3*$^{-/-}$ MECs, by treating cells with GSK3β inhibitor (SB-216763) or two distinct E-cadherin blocking antibodies (DECMA-1 and ECCD-1). As expected with GSK3β inhibition, SB-216763 treatment elevated *Ccnd1* and *Mmp7* gene expression in WT MECs (Fig. 6A,B). With respect to blocking E-cadherin, we observed a decrease in *Ccnd1* expression but an increase in *Mmp7* expression suggesting that perturbations in E-cadherin and GSK3β produce selective gene response in WT MECs. We then compared GSK3β- or E-cadherin-inhibitor-treated *Timp3*$^{-/-}$ with untreated *Timp3*$^{-/-}$ MECs, and observed significant elevation of *Ccnd1* and *Mmp7* expression following GSK3β inhibition, but not after E-cadherin blocking (Fig. 6A,B). This suggests that although WT MECs readily respond to GSK3β and E-cadherin blocking, *Timp3*$^{-/-}$ cells are refractory to E-cadherin-interfering antibodies.

Western blot analyses of these conditions indicated that dephosphorylated β-catenin levels rise in response to both GSK3β and E-cadherin inhibition in WT MECs (Fig. 6C). However, it produced little effect in *Timp3*$^{-/-}$ MECs, when compared with their respective no treatment controls that already have higher levels of this pool of β-catenin (Fig. 6C). It might be that the basal dephosphorylated β-catenin levels in *Timp3*$^{-/-}$ MECs represent the saturated levels for this protein

and/or that the canonical Wnt pathway is altered in *Timp3*$^{-/-}$ MECs. The levels of GSK3β activity, Frizzled receptor and Dishevelled remain to be studied.

TIMP3 expression influences mammary ductal elongation

The metalloproteinase axis and β-catenin are important factors in mammary gland development (Fata et al., 2004). MMP2, MMP3 and TIMP1 have been shown to affect ductal elongation and the branching propensity of the pubertal mouse mammary gland (Fata et al., 1999; Wiseman et al., 2003). Overexpression of stabilized β-catenin in the mammary luminal epithelium induces precocious lobuloalveolar differentiation, such that a virgin gland resembles a pregnant gland (Imbert et al., 2001). When stabilized β-catenin is overexpressed in the mammary gland myoepithelium, the virgin gland shows precocious lateral bud formation (Teuliere et al., 2005). We examined virgin mammary morphogenesis through whole mount analysis spanning 20-80 days in *Timp3*$^{-/-}$ and WT mammary glands. The rudimentary ductal tree was comparable at day 20 in the two groups, which was followed by a doubling in ductal length in *Timp3*$^{-/-}$ females by day 30. The accelerated ductal elongation was maintained at day 40 (Fig. 7A,B). This phenotype upon loss of TIMP3 function was transient because the average length of mammary ducts was equivalent in *Timp3*$^{-/-}$ and WT mammary glands by 60 and 80 days of age (data not shown). The number of ducts and branches per duct, which were measured in the area proximal to the lymph node (Wiseman et al., 2003), was not significantly different between *Timp3*$^{-/-}$ and WT mice aged 30 and 40 days (Fig. 7C,D). Next, we performed gain-of-function experiments in WT glands by implanting slow-release TIMP3-containing pellets. Parallel experiments included all four TIMPs to gain a comparative understanding of individual TIMP effects on mammary morphogenesis. We observed that TIMP1, TIMP3 and TIMP4 inhibited ductal elongation whereas TIMP2 exerted the opposite effect and promoted ductal elongation (Fig. 7E,F).

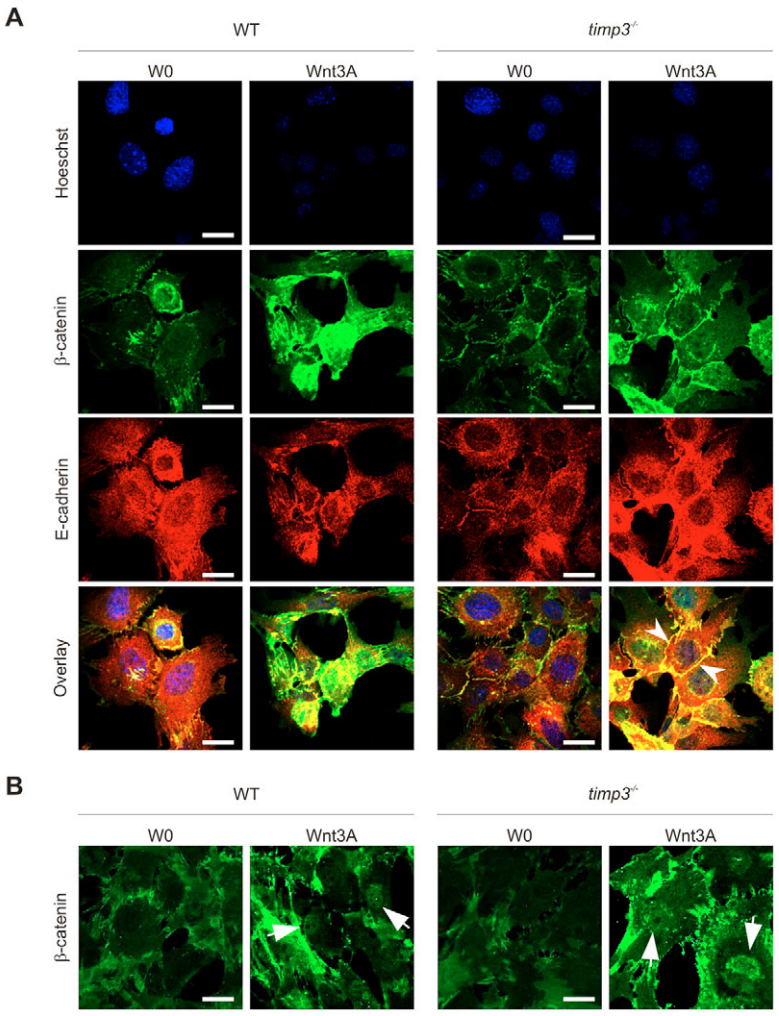

Fig. 3. Increased cell surface distribution of β-catenin in *Timp3*$^{-/-}$ MECs following Wnt3A stimulation. (A) Representative confocal images from three different experiments. The colocalization of E-cadherin and β-catenin was examined following treatment with W0 (control ligand) and Wnt3A in WT and *Timp3*$^{-/-}$ MECs. (B) Representative confocal images of WT and *Timp3*$^{-/-}$ mouse embryo fibroblasts treated with Wnt3A or control ligand. Arrows indicate nuclear localization of β-catenin; arrowheads indicate increased membrane colocalization of E-cadherin and β-catenin. Bars, 20 μm.

Discussion

Our study uncovers a novel connection between the metalloproteinase inhibitory activity of TIMP3 and β-catenin signaling in primary mammary epithelial cells. The loss of TIMP3 results in increased dephosphorylated β-catenin levels, elevated transcriptional activity and significant alterations in specific β-catenin target genes. Further, inhibitors of GSK3β and E-cadherin differentially affect *Ccnd1* and *Mmp7* gene expression revealing a complex relationship between TIMP3, β-catenin and specific downstream genes. Although WT epithelial cells readily respond to GSK3β and E-cadherin inhibition, *Timp3*$^{-/-}$ cells are refractory to E-cadherin-blocking antibodies. Fig. 8 presents changes in β-catenin and downstream gene responses as a function of TIMP3 loss, as well as following the manipulations of metalloproteinase activity, canonical Wnt signaling pathway and E-cadherin blocking. The responses to exogenous Wnt on *Timp3*$^{-/-}$ cells remain to be further investigated with respect to β-catenin transcriptional activity and cell adhesion. In vivo, TIMP3 levels are important for ductal elongation during pubertal mammary morphogenesis.

With loss of TIMP3, epithelial cells showed an increase in total and dephosphorylated β-catenin levels along with greater β-catenin transcriptional reporter activity, compared with that in fibroblasts. When β-catenin transcriptional targets were investigated in TIMP3-deficient cells, we found no difference in *Myc* mRNA levels, whereas *Mmp7* mRNA was substantially elevated and *Ccnd1* mRNA was significantly reduced. The *Mmp7* gene was not expressed in MEFs, which is consistent

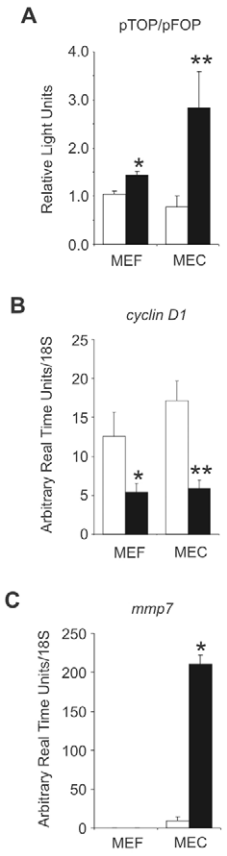

Fig. 4. Increased transcriptional activity and selective gene response in *Timp3*$^{-/-}$ MECs. (A) β-catenin transcriptional activity was measured using pTOPFLASH/pFOPFLASH luciferase reporter assay and was significantly higher in both *Timp3*$^{-/-}$ MEFs (**P*=0.001; □, WT; ■, *Timp3*$^{-/-}$) and *Timp3*$^{-/-}$ MECs (***P*=0.01) compared with their respective WT controls. Relative light unit values were normalized against a β-galactosidase transfection control. (B,C) Expression of β-catenin target genes was assessed by TaqMan real-time PCR and are normalized to 18S RNA values for each sample. (B) *Ccnd1* mRNA level was significantly lower in both *Timp3*$^{-/-}$ MEFs (**P*=0.05) and *Timp3*$^{-/-}$ MECs (***P*=0.002). (C) *Mmp7* mRNA level was significantly increased only in *Timp3*$^{-/-}$ MECs (**P*=0.005). Two-tailed, Student's *t*-test was performed to assess statistical significance between WT and *Timp3*$^{-/-}$. Values are expressed as mean ± s.e.m. of three independent experiments.

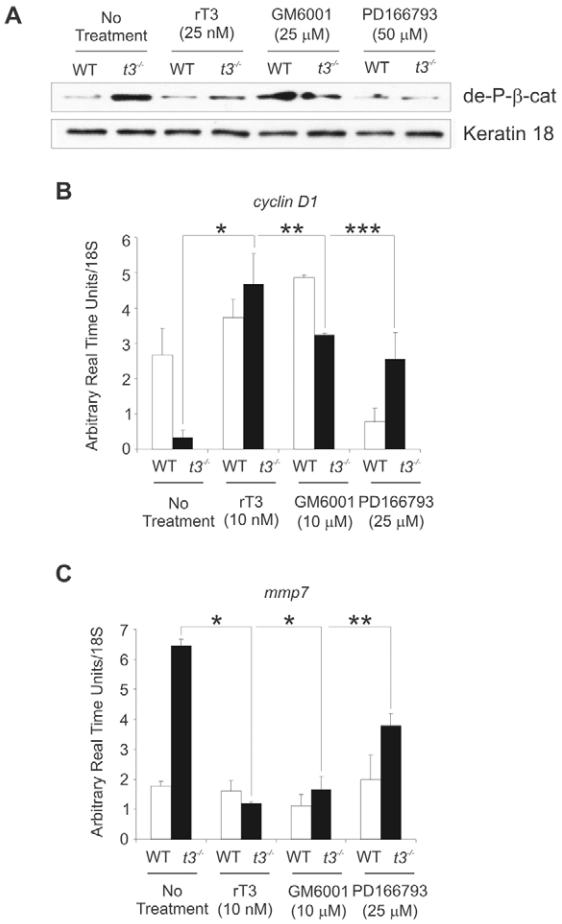

Fig. 5. Metalloproteinase inhibitors decrease β-catenin signaling in *Timp3*$^{-/-}$ cells. (A) Western blots of whole-cell lysates from WT and *Timp3*$^{-/-}$ MECs revealed that metalloproteinase inhibition with recombinant TIMP3 (25 μM), GM6001 (25 μM) and PD166793 (50 μM) for 24 hours returned dephosphorylated β-catenin to levels comparable to the WT. (B) The reduction in *Ccnd1* gene expression in *Timp3*$^{-/-}$ MECs was reversed by 24-hour treatment with recombinant TIMP3 (10 μM) (**P*<0.001), GM6001 (10 μM) (***P*<0.01) and PD166793 (25 μM) (****P*<0.05) when compared with the untreated control. (C) The increase in *Mmp7* gene expression in *Timp3*$^{-/-}$ MECs was significantly mitigated by treatment with recombinant TIMP3 (10 μM) (**P*<0.001), GM6001 (10 μM) (**P*<0.001) and PD166793 (25 μM) (***P*<0.05) for 24 hours. ANOVA was performed to assess the statistical significance between WT and *Timp3*$^{-/-}$ cells. Values are expressed as mean ± s.e.m. of three independent experiments.

with its epithelial specificity (Wilson et al., 1995). Dysregulated β-catenin levels are associated with MMP7 expression in breast and colon cancers (Mylona et al., 2005; Ougolkov et al., 2002). Transgenic overexpression of MMP7 in the mammary gland results in mammary hyperplasia and selects for apoptosis-resistant mammary epithelial cells (Rudolph-Owen et al., 1998; Vargo-Gogola et al., 2002). Consistent with this, mammary ductal elongation was transiently accelerated in *Timp3* null pubertal females. Regarding *Ccnd1* gene expression, both MEFs and MECs showed reduction in the absence of TIMP3. The requirement of cyclin D1 in β-catenin signaling has been studied both in context of mammary tumorigenesis and mammary development. Expression of stabilized β-catenin induces mammary adenocarcinomas that are unexpectedly enhanced by cyclin D1 deficiency (Rowlands et al., 2003). On the other hand, cyclin D1 is essential for alveologenesis induced by β-catenin stabilization (Rowlands et al., 2003). Thus, β-catenin and cyclin D1 play a complex role in the mammary gland. Understanding the functional significance of TIMP3 on this relationship, as well the role of TIMP3 in the interaction of β-catenin and MMP7, requires further investigation.

We have reported that TIMP1 functions in its capacity as a metalloproteinase inhibitor to maintain cell-cell contact in fibroblasts (Ho et al., 2001). Similar to *Timp1* downregulated fibroblasts, *Timp3*$^{-/-}$ MEFs aggregated poorly, suggesting a defect in cell-cell contact. However, unlike *Timp1*, MEFs deficient in *Timp3* had normal levels of several cadherins and

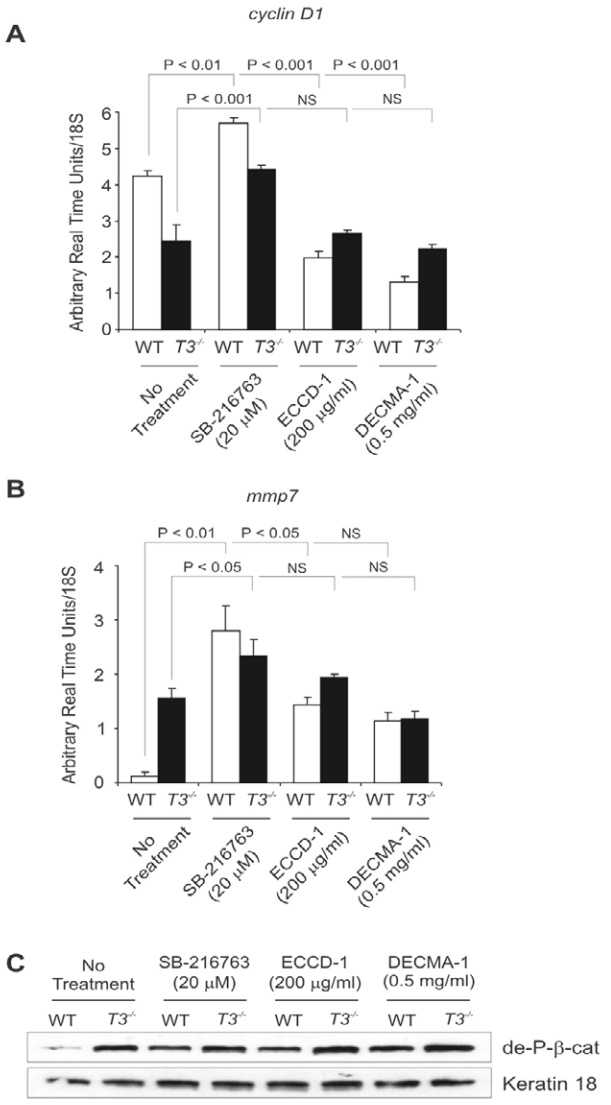

Fig. 6. Effects of GSK3β and E-cadherin inhibitors on *Ccnd1* and *Mmp7* gene expression. (A,B) WT (□) and *Timp3*$^{-/-}$ (■) MECs were treated with the GSK3β inhibitor, SB-216763 (20 mM for 24 hours) and the E-cadherin blocking antibodies, ECCD-1 (200 mg/ml for 6 hours) and DECMA-1 (0.5 mg/ml for 6 hours). Expression of β-catenin target genes, *Ccnd1* (A) and *Mmp7* (B) was assessed by TaqMan real-time PCR and normalized to 18S RNA values. (A) Inhibition of GSK3β led to an overall increase in *Ccnd1* mRNA levels in WT and *Timp3*$^{-/-}$ MECs, but still showed significantly lower levels in *Timp3*$^{-/-}$ MECs compared with treated WT MECs. Similarly, treatment with SB-216763 of WT and *Timp3*$^{-/-}$ MECs generally increased *Mmp7* mRNA levels. Upon GSK3β inhibition WT and *Timp3*$^{-/-}$ expression of *Mmp7* became comparable (B). E-cadherin inhibition using two different blocking antibodies decreased *Ccnd1* and increased *Mmp7* mRNA levels in treated WT MECs but had no effect on *Timp3*$^{-/-}$ MECs. (C) Treatment with SB-216763, ECCD-1 and DECMA-1 elevated WT MEC levels of dephosphorylated β-catenin (de-P-β-cat) to those of untreated *Timp3*$^{-/-}$ MEC levels. The β-catenin levels in *Timp3*$^{-/-}$ MECs may be maximal as treatment with the GSK3β inhibitor did not further increase dephosphorylated β-catenin levels. ANOVA was performed to assess statistical significance between WT and *Timp3*$^{-/-}$ cells. Values are expressed as mean ± s.e.m. of three independent experiments.

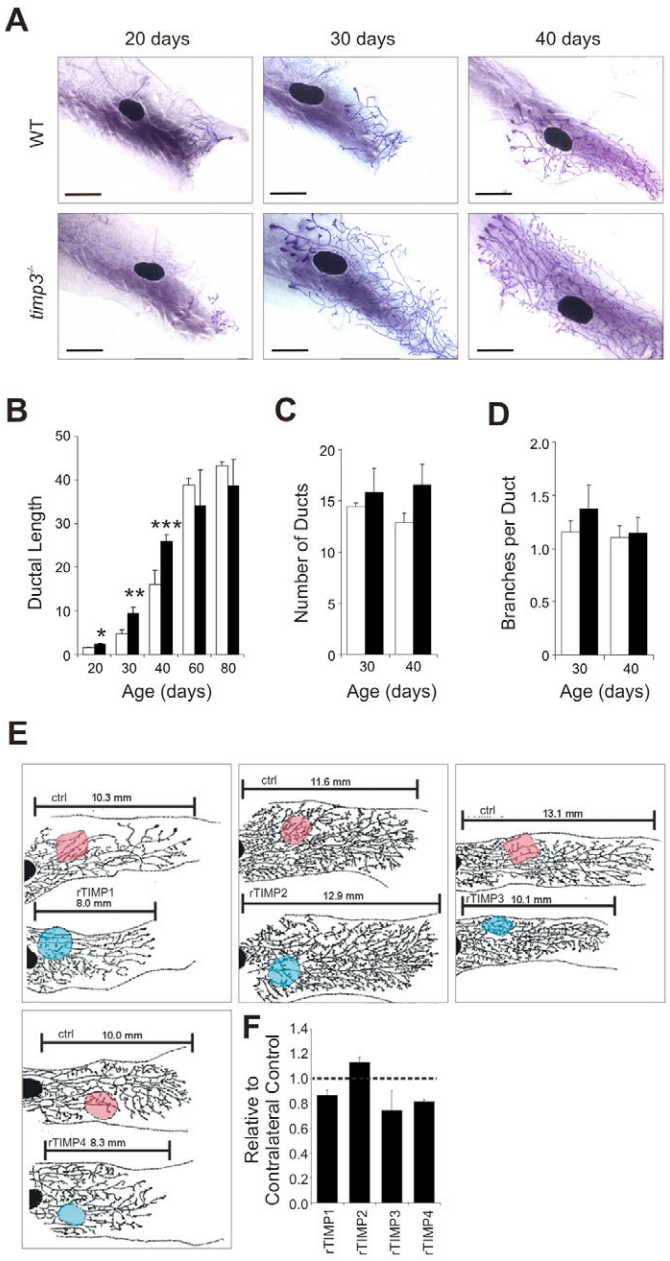

Fig. 7. Accelerated mammary ductal elongation in *Timp3*$^{-/-}$ virgin glands. (A) Representative images of mammary gland whole mounts (n=4) during pubertal morphogenesis in WT and *Timp3*$^{-/-}$ mammary glands. Bars, 1 mm. A significant increase was seen in (B) ductal length at 20 (*P=0.016), 30 (**P=0.019) and 40 (***P=0.022) days of age. There were no statistically significant differences in (C) numbers of ducts and (D) branches per duct between WT and *Timp3*$^{-/-}$ mammary glands (□, WT; ■, *Timp3*$^{-/-}$). (E) Implantation of recombinant TIMP-containing pellets (blue) or control pellets (red) into contralateral WT glands revealed effects of individual TIMPs on mammary morphogenesis. (F) TIMP1, TIMP3 and TIMP4 retarded mammary ductal elongation by 12% (P=0.04), 25% (P=0.07) and 19% (P=0.002), relative to contralateral glands, respectively. TIMP2 promoted ductal elongation by 13% relative to contralateral gland (P<0.05). Student's t-test was performed to assess statistical significance between WT and *Timp3*$^{-/-}$ mammary glands. Values are expressed as mean ± s.e.m.

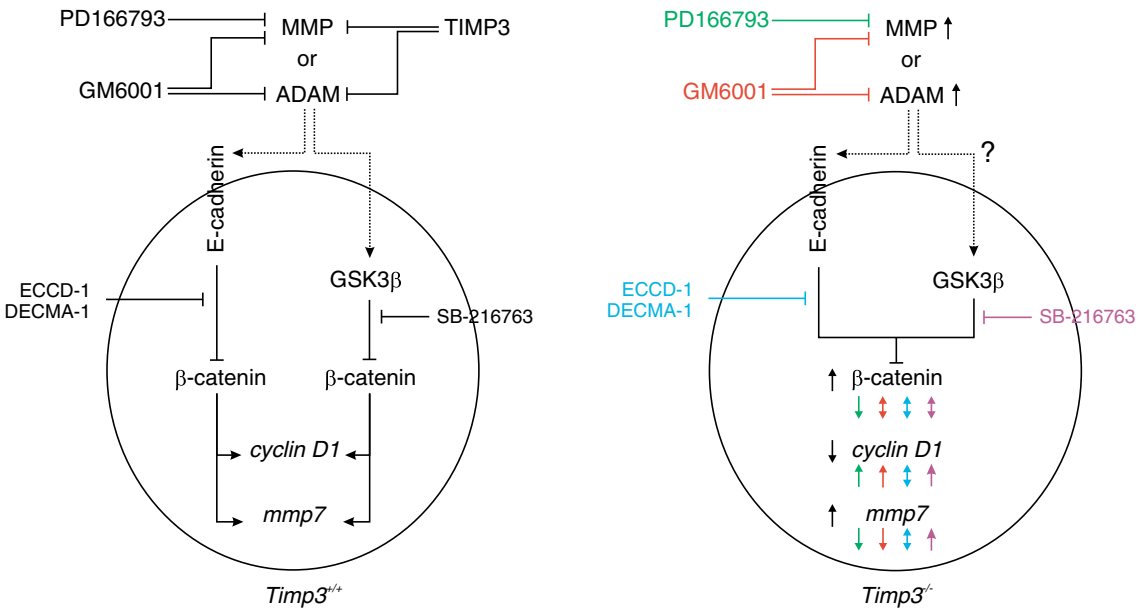

Fig. 8. A schematic outlining the relationships between metalloproteinases, metalloproteinase inhibitors and regulators of β-catenin signaling. In *Timp3*$^{+/+}$ setting, E-cadherin and GSK3β influence the cytoplasmic pool of β-catenin, which can translocate into the nucleus and function as transcriptional co-activator for target genes, *Ccnd1* (cyclin D1) and *Mmp7*. We propose that MMP and ADAM activity operates upstream of E-cadherin and GSK3β through yet unidentified mechanisms to influence the signaling pool of β-catenin. In *Timp3* deficient mammary epithelial cells, β-catenin signaling activity is increased, leading to selective gene responses. These arise in a GSK3β-dependent manner and are not influenced by E-cadherin blocking. (↑, increased expression or activity; ↓, decreased expression or activity; ↕, unchanged expression or activity.)

the expected colocalization with β-catenin. These differences might stem from the intrinsically different properties of the two inhibitors. TIMP3 is ECM-bound whereas TIMP1 is freely soluble upon secretion (Pavloff et al., 1992; Yu et al., 2000). The inhibitory capacity of TIMP3 extends to ADAM10, ADAM17 (Black et al., 1997; Lee et al., 2001; Mohammed et al., 2004), ADAM12S (Loechel et al., 2000), ADAMTS4 and ADAMTS5 (Kashiwagi et al., 2001). Additionally, MT1-MMP required for pro-MMP2 activation at the cell surface (Strongin et al., 1995) is inhibited only by TIMP3 (English et al., 2006; Lee et al., 2003). Several of these proteases shed cell surface proteins, and as such, TIMP3 deficiency might allow processing of molecules other than cadherins, which are involved in aggregation (Kheradmand and Werb, 2002). Treatment with metalloproteinase inhibitors led to equivalent levels of dephosphorylated β-catenin in *Timp3*$^{-/-}$ and WT MECs. Specifically, treatment with the broad-spectrum inhibitor GM6001 raised dephosphorylated β-catenin in WT MECs, whereas the MMP-specific inhibitor PD166793 reduced this signaling pool of β-catenin in *Timp3*$^{-/-}$ MECs. This suggests a complex interaction between specific metalloproteinase candidates, the individual TIMPs and intracellular signaling pathways. For example, ADAM10, which can be inhibited by both TIMP1 and TIMP3 (Amour et al., 2000), alters the stability of N-cadherin and E-cadherin on the cell surface, β-catenin subcellular localization and *Ccnd1* gene expression (Maretzky et al., 2005; Reiss et al., 2005). ADAM-specific inhibitors are needed to more comprehensively delineate the role of distinct metalloproteinase axes in β-catenin signaling.

Ductal morphogenesis provides a developmental window to assess the effects of different molecules on epithelial cell proliferation and branching morphogenesis. Often these effects are transient, as has been reported for growth factors such as IGF2 (Moorehead et al., 2003), metalloproteinases and TIMPs such as MMP2, MMP3 (Wiseman et al., 2003) and TIMP1 (Fata et al., 1999), and oncogenes such as ErbB2 (Mukherjee et al., 2000). The metalloproteinase proteolytic axis is an important regulator of mammary epithelium morphogenesis during the pubertal developmental stage of the gland. MMP2 and MMP3 differentially regulate epithelial branching in a site- and stage-specific manner during virgin morphogenesis (Wiseman et al., 2003). Mammary epithelium-specific TIMP1 reduction leads to increased epithelial proliferation and ductal expansion (Fata et al., 1999). ADAM17 has been reported to regulate amphiregulin bioactivity in the developing mammary gland (Sternlicht et al., 2005). Here we show through loss-of-function and gain-of-function studies that TIMP3 is a negative regulator of pubertal ductal elongation. This property is shared by TIMP1 and TIMP4, and not by TIMP2, further supporting the specific requirements of key metalloproteinases during pubertal development of the mammary gland.

Timp3$^{-/-}$ MECs had increased β-catenin transcriptional activity and higher β-catenin nuclear levels have been described in breast cancer (Lin et al., 2000). Transgenic overexpression of stabilized β-catenin in the mammary gland results in mammary hyperplasia and adenocarcinoma (Imbert et al., 2001; Teuliere et al., 2005) and MMP7 overexpression is also conducive to mammary tumorigenesis (Rudolph-Owen et al., 1998; Vargo-Gogola et al., 2002). Beyond breast cancer, aberrant β-catenin signaling and activity is also implicated in mesenchymal tumors such as aggressive fibromatosis and

synovial sarcomas (Cheon et al., 2002; Ng et al., 2005). Thus, TIMP3 deficiency and the pathways it alters may promote tumorigenesis. However, it also remains possible that TIMP3 deficiency will suppress tumorigenesis. *Timp3*$^{-/-}$ MECs showed resistance to EGTA-induced detachment which may underlie the formation of more stable cell-cell contact. In epithelial cancers, independence from contact-inhibited growth, loss of cell-cell contact, and acquisition of mesenchymal characteristics have been associated with a propensity for invasion and metastasis (Savagner, 2001; Thiery, 2002; Vincent-Salomon and Thiery, 2003). Furthermore, upon Wnt3A treatment, we observed β-catenin mobilization to the cell membrane rather than into the nucleus. Hinck et al. made a similar observation in a pituitary tumor cell line and epithelial cells treated with Wnt1, where increased levels of β-catenin were observed in the membrane fractions of PC12, C57MG and MDCK cells (Bradley et al., 1993; Giarre et al., 1998; Hinck et al., 1994). Subsequent studies demonstrated that the switch that drives β-catenin localization is determined by its binding partners such as α-catenin and BCL9-2 (Brembeck et al., 2004; Gottardi and Gumbiner, 2004), which can be conceivably altered in the TIMP3 deficient state. Additionally, we observed a specific reduction in *Ccnd1* gene expression in *Timp3*$^{-/-}$ MECs which has the capacity to affect mammary tumorigenesis (Sutherland and Musgrove, 2002; Yu et al., 2001). We are currently investigating mammary tumorigenesis in relation to *Timp3* deficiency.

In this report, we demonstrate that the extracellular metalloproteinase inhibitor TIMP3 affects intracellular signaling pathways that involve β-catenin. TIMP3 acts in a cell-type-specific manner to influence selective gene expression and cell behavior. TIMP3 loss affects its target genes through MMP and ADAM proteolytic activity. Our work expands the scope of β-catenin regulation beyond canonical Wnt signaling by demonstrating the importance of pericellular proteolytic regulation of this pathway.

Materials and Methods

Primary cell cultures

MEFs were isolated from individual embryos at day E14.5 from *Timp3*$^{+/-}$ breeding pairs. After removing the heads and internal organs, the embryos were finely macerated to 1-mm^3 pieces. The tissue was then incubated in trypsin-EDTA for 30 minutes. After several washes with PBS the cells were cultured in DMEM supplemented with 10% FBS. MEFs were genotyped for TIMP3 status. Five WT and five *Timp3*$^{-/-}$ MEF clones from different parents were used for these studies. MEF cultures were used for up to six passages only.

Primary MECs were harvested from all five pairs of mammary glands from 6- to 8-week-old virgin mice. Each MEC preparation consisted of five female mice. The glands were finely macerated to 1-mm^3 pieces and further dispersed with digestion media (DMEM/Ham's F12 1:1 media supplemented with 60 U/ml nystatin, 100 μg/ml gentamycin, 100 U/ml hyaluronidase and 2 mg/ml collagenase) treatment for 3 hours at 100 r.p.m. at 37°C. The tissue was filtered through a 40 μm filter and the cells were washed five times with PBS supplemented with 5% adult bovine serum. Finally, the cells were cultured in growth media (DMEM/Ham's F12 1:1 media supplemented with 10 μg/ml insulin, 5 ng/ml EGF, 1 mg/ml BSA, 5 μg/ml linoleic acid complex, 20 U/ml nystatin, 50 μg/ml gentamycin and 2% adult bovine serum) and were purified and passaged using differential dispase treatment. MEC cultures were used for up to six passages only. Epithelial cell purity of the primary MEC cultures was assessed by measuring cytokeratin 18 (epithelial cell-specific marker) and vimentin (fibroblast-specific marker) levels.

Aggregation assay

Aggregation assay was performed as previously described (Ho et al., 2001), except that cells were incubated for 15, 30, 45 and 60 minutes.

Lysate preparation and immunoblotting

For whole-cell lysate preparations, MEFs and MECs were transferred to RIPA buffer supplemented with protease and phosphatase inhibitors (1 mM sodium vanadate, 1 mM EDTA, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 5 μg/ml aprotinin) and kept on ice for 30 minutes. Lysates were centrifuged at 10,000 ***g*** for 10 minutes and the supernatant was collected as whole-cell lysates. For nuclear extracts, cells were collected and incubated in Buffer A (0.65% NP-40, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 minutes on ice. The cells were centrifuged at 83,250 ***g*** for 1 minute. The pellet was incubated with Buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 30 minutes on ice with frequent mixing. After the cell mixture was centrifuged at 83,250 ***g*** for 5 minutes, the supernatant was discarded and the pellet (nuclear fraction) was dissolved in SDS buffer. Lysates were quantified using D_c Bio-Rad reagent according to the manufacturer's instructions.

For whole-cell lysates, 30 μg protein was separated in 8% SDS-PAGE gels. For subcellular fractions, 2 μg protein was loaded. Primary antibodies against the following targets were used at the indicated dilutions: anti-β-catenin (Transduction Labs), 1:2000; anti-dephosphorylated β-catenin (A.G. Scientific), 1:1000; anti-pan-cadherin, (Santa Cruz), 1:1000; anti-E-cadherin (Transduction Labs), 1:1000; anti-N-cadherin (Transduction Labs), 1:500; anti-H-cadherin (Santa Cruz), 1:1000; anti-T-cadherin (Santa Cruz), 1:1000. Amido Black staining was performed by incubating newly transferred blots with diluted Amido Black stain (0.001% Amido Black in 5:4:1 mix of methanol:water:glacial acetic acid) for 15 seconds. Silver staining (Bio-Rad) of SDS-PAGE gels loaded with 2 μg protein was performed according to the manufacturer's instructions. Silver and Amido Black staining were chosen as the least biased loading controls as other traditional loading controls (tubulin and GAPDH) showed variability in mammary epithelial cell protein preparations. Densitometric analyses on western blots were performed using Northern Eclipse image analysis software.

EGTA-induced detachment by Ca^{2+} chelation

At 70% confluency, cells were serum starved for 6 hours. The media was replaced with Ca^{2+} chelation medium (serum-free medium supplemented with 4 mM EGTA and 1 mM $MgCl_2$) and the cells were incubated for 0, 0.5, 1, 2, 4 and 6 hours. Both viable and dead cells were counted by Trypan Blue exclusion in supernatants that were collected at each time point. Cells that remained attached were incubated with Hoechst 33342 (0.05 μg/ml), fixed with ice-cold methanol for 10 minutes and analyzed using fluorescence microscopy.

Wnt3A treatment

Control (W0) and Wnt3A ligand were collected from the supernatant of L cells that were stably transfected with mock (W0) or Wnt3A cDNA (ATCC) and selected with G418 (Willert et al., 2003). MEFs and MECs were serum starved overnight and treated with either mock supernatant or Wnt3A-containing supernatants for 4 hours.

Inhibitor treatments

Primary MECs were treated with the following metalloproteinase inhibitors for 6 and 24 hours: GM6001 (Calbiochem) at 10 μM and 25 μM, recombinant TIMP3 (R&D Systems) at 10 nM and 25 nM, and PD166793 (Pfizer) at 25 μM and 50 μM, following previously published reports (English et al., 2006; Kassiri et al., 2005; Spinale et al., 1999). For each time point, total RNA and total protein were collected. Primary MECs were treated with the GSK3β inhibitor, SB-216763 (Sigma) at 10 mM and 20 mM for 24 hours, or the E-cadherin blocking antibodies, ECCD-1 (Chemicon; 200 mg/ml) and DECMA-1 (Chemicon; 0.5 mg/ml) for 1 and 6 hours.

Immunocytochemistry

Cells were grown on poly-L-lysine-treated glass coverslips to 70% confluency, at which point they were left untreated, or treated with EGTA, W0 or Wnt3A. After the respective incubation times, cells were fixed and permeabilized with ice-cold methanol for 10 minutes. After several PBS washes, the cells were blocked with goat serum for 1 hour at room temperature. Primary antibodies (anti-pan-cadherin, anti-E-cadherin, anti-N-cadherin and anti-β-catenin; all at 1:500) were incubated overnight at 4°C in a humidified chamber. After several washes, FITC- or Rhodamine-conjugated secondary antibodies were incubated for 30 minutes at room temperature. Secondary antibodies were also used alone as controls. Finally the cells were incubated with Hoechst 33342 (0.05 μg/ml) for 5 minutes. The glass coverslips were mounted onto slides using Glycergel and sealed with nail polish. The cells were analyzed using Zeiss LSM510 confocal microscopy and associated software. Exposure times were kept constant throughout all image capture to allow for qualitative signal intensity comparisons.

β-catenin transcriptional activity assay

MEFs and MECs were seeded onto six-well dishes and transfected with pCMV-β-galactosidase (0.25 μg) and either pTOPFLASH (3 μg) or pFOPFLASH vectors (3 μg) using FuGene 6 (Roche) transfection reagent according to the manufacturer's protocol. The cells were transfected and lysates were prepared 48 hours later using 1× reporter lysis buffer (Promega). Lysates were analyzed using a luminometer. Transfection efficiency was analyzed by assessing β-galactosidase activity and all pTOPFLASH and pFOPFLASH values were normalized to β-galactosidase activity.

Briefly, lysates were incubated with 1× ONPG, 1× Mg^{2+} and 0.1 M sodium phosphate. The reaction was quenched using 1 M sodium carbonate and the absorbance was analyzed at 420 nm.

Quantitative TaqMan real-time RT-PCR

RNA expression levels for the genes reported in this study were quantified by real-time TaqMan RT-PCR using the ABI Prism 7700 sequence detection system as described previously (Young et al., 2002). Briefly, 1 μg total RNA was reverse transcribed using random hexamers. TaqMan reactions were conducted in 96-well plates using cDNA, 12.5 μl of 2× TaqMan universal PCR master mix, 100 μM probe and 200 μM of each primer, and water to a final volume of 25 μl. The levels of *Mmp7* were measured using published primers and probes (Nuttall et al., 2004). We used the PrimerExpress software to design primers and probes for the following genes: *Myc*, forward primer: 5′-GACAAGAGGCGGACACACAAC-3′, reverse primer: 5′-GCTGCGCTTCAGCTCGTT-3′, probe: 5′-FAM-TTGGAACGTCAGAGGAG-TAMRA-3′; *Ccnd1*, forward primer: 5′-CAGGCGTTCAGAACCAGATTC-3′, reverse primer: 5′-CCCTCCAATAGCAGCGAAAAC-3′, probe: 5′-FAM-ATTGCTTTGTATCTTTCACG-TAMRA-3′. We used 18S rRNA as an endogenous control and all mRNA levels were normalized to the 18S controls.

Experimental mice and mammary gland analyses

At 20, 30, 40, 60 and 80 days of age, five independent WT and *Timp3*$^{-/-}$ females, each belonging to a comparable litter size, were used for these analyses. The fourth inguinal mammary gland was collected from each female. Whole mount procedures were performed as previously described (Fata et al., 1999). Ductal length, number of ducts, and number of branches per duct were analyzed as described in detail by Wiseman et al. (Wiseman et al., 2003). Implantation of slow-release pellets containing recombinant TIMPs or PBS control into the fourth inguinal glands was performed as previously described (Fata et al., 1999).

We would like to thank Lilliana Attisano for help with β-catenin analyses. This work has been supported by funding from Canadian Institutes of Health Research to R.K. C.V.H. was a recipient of National Science and Engineering Research Council studentship and currently holds a National Cancer Institute of Canada studentship. Z.K. currently holds Heart and Stroke post-doctoral fellowship.

References

Ahonen, M., Poukkula, M., Baker, A. H., Kashiwagi, M., Nagase, H., Eriksson, J. E. and Kahari, V. M. (2003). Tissue inhibitor of metalloproteinases-3 induces apoptosis in melanoma cells by stabilization of death receptors. *Oncogene* **22**, 2121-2134.

Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Docherty, A. J. and Murphy, G. (2000). The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. *FEBS Lett.* **473**, 275-279.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**, 729-733.

Bond, M., Murphy, G., Bennett, M. R., Newby, A. C. and Baker, A. H. (2002). Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. *J. Biol. Chem.* **277**, 13787-13795.

Bradley, R. S., Cowin, P. and Brown, A. M. (1993). Expression of Wnt-1 in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. *J. Cell Biol.* **123**, 1857-1865.

Brembeck, F. H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M. and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. *Genes Dev.* **18**, 2225-2230.

Chapman, R. E., Scott, A. A., Deschamps, A. M., Lowry, A. S., Stroud, R. E., Ikonomidis, J. S. and Spinale, F. G. (2003). Matrix metalloproteinase abundance in human myocardial fibroblasts: effects of sustained pharmacologic matrix metalloproteinase inhibition. *J. Mol. Cell. Cardiol.* **35**, 539-548.

Cheon, S. S., Cheah, A. Y., Turley, S., Nadesan, P., Poon, R., Clevers, H. and Alman, B. A. (2002). beta-Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. *Proc. Natl. Acad. Sci. USA* **99**, 6973-6978.

Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P. and Matrisian, L. M. (1999). The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene* **18**, 2883-2891.

Davies, G., Jiang, W. G. and Mason, M. D. (2001). Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. *Clin. Cancer Res.* **7**, 3289-3297.

Egeblad, M. and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2**, 161-174.

English, J. L., Kassiri, Z., Koskivirta, I., Atkinson, S. J., Di Grappa, M., Soloway, P. D., Nagase, H., Vuorio, E., Murphy, G. and Khokha, R. (2006). Individual timp deficiencies differentially impact pro-MMP-2 activaiton. *J. Biol. Chem.* **15**, 10337-10346.

Fata, J. E., Leco, K. J., Moorehead, R. A., Martin, D. C. and Khokha, R. (1999). Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary development. *Dev. Biol.* **211**, 238-254.

Fata, J. E., Leco, K. J., Voura, E. B., Yu, H. Y., Waterhouse, P., Murphy, G., Moorehead, R. A. and Khokha, R. (2001). Accelerated apoptosis in the Timp-3-deficient mammary gland. *J. Clin. Invest.* **108**, 831-841.

Fata, J. E., Werb, Z. and Bissell, M. J. (2004). Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res.* **6**, 1-11.

George, S. J. and Dwivedi, A. (2004). MMPs, cadherins, and cell proliferation. *Trends Cardiovasc. Med.* **14**, 100-105.

Giarre, M., Semenov, M. V. and Brown, A. M. (1998). Wnt signaling stabilizes the dual-function protein beta-catenin in diverse cell types. *Ann. N. Y. Acad. Sci.* **857**, 43-55.

Gottardi, C. J. and Gumbiner, B. M. (2004). Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *J. Cell Biol.* **167**, 339-349.

Haertel-Wiesmann, M., Liang, Y., Fantl, W. J. and Williams, L. T. (2000). Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. *J. Biol. Chem.* **275**, 32046-32051.

Hatsell, S., Rowlands, T., Hiremath, M. and Cowin, P. (2003). Beta-catenin and Tcfs in mammary development and cancer. *J. Mammary Gland Biol. Neoplasia* **8**, 145-158.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

Hinck, L., Nelson, W. J. and Papkoff, J. (1994). Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. *J. Cell Biol.* **124**, 729-741.

Ho, A. T., Voura, E. B., Soloway, P. D., Watson, K. L. and Khokha, R. (2001). MMP inhibitors augment fibroblast adhesion through stabilization of focal adhesion contacts and up-regulation of cadherin function. *J. Biol. Chem.* **276**, 40215-40224.

Hojilla, C. V., Mohammed, F. F. and Khokha, R. (2003). Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. *Br. J. Cancer* **89**, 1817-1821.

Imbert, A., Eelkema, R., Jordan, S., Feiner, H. and Cowin, P. (2001). Delta N89 beta-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *J. Cell Biol.* **153**, 555-568.

Kashiwagi, M., Tortorella, M., Nagase, H. and Brew, K. (2001). TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). *J. Biol. Chem.* **276**, 12501-12504.

Kassiri, Z., Oudit, G. Y., Sanchez, O., Dawood, F., Mohammed, F. F., Nuttall, R. K., Edwards, D. R., Liu, P. P., Backx, P. H. and Khokha, R. (2005). Combination of tumor necrosis factor-alpha ablation and matrix metalloproteinase inhibition prevents heart failure after pressure overload in tissue inhibitor of metalloproteinase-3 knock-out mice. *Circ. Res.* **97**, 380-390.

Kheradmand, F. and Werb, Z. (2002). Shedding light on sheddases: role in growth and development. *BioEssays* **24**, 8-12.

Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G. and Denhardt, D. T. (1989). Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. *Science* **243**, 947-950.

Lambert, E., Dasse, E., Haye, B. and Petitfrere, E. (2004). TIMPs as multifacial proteins. *Crit. Rev. Oncol. Hematol.* **49**, 187-198.

Leco, K. J., Khokha, R., Pavloff, N., Hawkes, S. P. and Edwards, D. R. (1994). Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* **269**, 9352-9360.

Lee, J. S., Ishimoto, A. and Yanagawa, S. (1999). Characterization of mouse dishevelled (Dvl) proteins in Wnt/Wingless signaling pathway. *J. Biol. Chem.* **274**, 21464-21470.

Lee, M. H., Knauper, V., Becherer, J. D. and Murphy, G. (2001). Full-length and N-TIMP-3 display equal inhibitory activities toward TNF-alpha convertase. *Biochem. Biophys. Res. Commun.* **280**, 945-950.

Lee, M. H., Rapti, M. and Murphy, G. (2003). Unveiling the surface epitopes that render tissue inhibitor of metalloproteinase-1 inactive against membrane type 1-matrix metalloproteinase. *J. Biol. Chem.* **278**, 40224-40230.

Lin, S. Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, B., Wen, Y., Pestell, R. G. and Hung, M. C. (2000). Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. USA* **97**, 4262-4266.

Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z. and Bissell, M. J. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J. Cell Biol.* **139**, 1861-1872.

Loechel, F., Fox, J. W., Murphy, G., Albrechtsen, R. and Wewer, U. M. (2000). ADAM 12-S cleaves IGFBP-3 and IGFBP-5 and is inhibited by TIMP-3. *Biochem. Biophys. Res. Commun.* **278**, 511-515.

Maretzky, T., Reiss, K., Ludwig, A., Buchholz, J., Scholz, F., Proksch, E., de Strooper, B., Hartmann, D. and Saftig, P. (2005). ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc. Natl. Acad. Sci. USA* **102**, 9182-9187.

McGuire, J. K., Li, Q. and Parks, W. C. (2003). Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. *Am. J. Pathol.* **162**, 1831-1843.

Mohammed, F. F., Smookler, D. S. and Khokha, R. (2003). Metalloproteinases, inflammation, and rheumatoid arthritis. *Ann. Rheum. Dis.* **62** Suppl. 2, ii43-ii47.

Mohammed, F. F., Smookler, D. S., Taylor, S. E., Fingleton, B., Kassiri, Z., Sanchez, O. H., English, J. L., Matrisian, L. M., Au, B., Yeh, W. C. et al. (2004). Abnormal TNF activity in Timp3–/– mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat. Genet.* **36**, 969-977.

Moon, R. T., Kohn, A. D., De Ferrari, G. V. and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5**, 691-701.

Moorehead, R. A., Hojilla, C. V., De Belle, I., Wood, G. A., Fata, J. E., Adamson, E. D., Watson, K. L., Edwards, D. R. and Khokha, R. (2003). Insulin-like growth factor-II regulates PTEN expression in the mammary gland. *J. Biol. Chem.* **278**, 50422-50427.

Mukherjee, S., Louie, S. G., Campbell, M., Esserman, L. and Shyamala, G. (2000). Ductal growth is impeded in mammary glands of C-neu transgenic mice. *Oncogene* **19**, 5982-5987.

Mylona, E., Kapranou, A., Mavrommatis, J., Markaki, S., Keramopoulos, A. and Nakopoulou, L. (2005). The multifunctional role of the immunohistochemical expression of MMP-7 in invasive breast cancer. *Apmis* **113**, 246-255.

Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483-1487.

Ng, T. L., Gown, A. M., Barry, T. S., Cheang, M. C., Chan, A. K., Turbin, D. A., Hsu, F. D., West, R. B. and Nielsen, T. O. (2005). Nuclear beta-catenin in mesenchymal tumors. *Mod. Pathol.* **18**, 68-74.

Noe, V., Fingleton, B., Jacobs, K., Crawford, H. C., Vermeulen, S., Steelant, W., Bruyneel, E., Matrisian, L. M. and Mareel, M. (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J. Cell Sci.* **114**, 111-118.

Nuttall, R. K., Sampieri, C. L., Pennington, C. J., Gill, S. E., Schultz, G. A. and Edwards, D. R. (2004). Expression analysis of the entire MMP and TIMP gene families during mouse tissue development. *FEBS Lett.* **563**, 129-134.

Ougolkov, A. V., Yamashita, K., Mai, M. and Minamoto, T. (2002). Oncogenic beta-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer. *Gastroenterology* **122**, 60-71.

Pavloff, N., Staskus, P. W., Kishnani, N. S. and Hawkes, S. P. (1992). A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. *J. Biol. Chem.* **267**, 17321-17326.

Puente, X. S., Sanchez, L. M., Overall, C. M. and Lopez-Otin, C. (2003). Human and mouse proteases: a comparative genomic approach. *Nat. Rev. Genet.* **4**, 544-558.

Reiss, K., Maretzky, T., Ludwig, A., Tousseyn, T., de Strooper, B., Hartmann, D. and Saftig, P. (2005). ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. *EMBO J.* **24**, 742-752.

Rothen-Rutishauser, B., Riesen, F. K., Braun, A., Gunthert, M. and Wunderli-Allenspach, H. (2002). Dynamics of tight and adherens junctions under EGTA treatment. *J. Membr. Biol.* **188**, 151-162.

Rowlands, T. M., Pechenkina, I. V., Hatsell, S. J., Pestell, R. G. and Cowin, P. (2003). Dissecting the roles of beta-catenin and cyclin D1 during mammary development and neoplasia. *Proc. Natl. Acad. Sci. USA* **100**, 11400-11405.

Rudolph-Owen, L. A., Cannon, P. and Matrisian, L. M. (1998). Overexpression of the matrix metalloproteinase matrilysin results in premature mammary gland differentiation and male infertility. *Mol. Biol. Cell* **9**, 421-435.

Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *BioEssays* **23**, 912-923.

Spinale, F. G., Coker, M. L., Krombach, S. R., Mukherjee, R., Hallak, H., Houck, W. V., Clair, M. J., Kribbs, S. B., Johnson, L. L., Peterson, J. T. et al. (1999). Matrix metalloproteinase inhibition during the development of congestive heart failure: effects on left ventricular dimensions and function. *Circ. Res.* **85**, 364-376.

Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J. P., Gray, J. W., Pinkel, D., Bissell, M. J. and Werb, Z. (1999). The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* **98**, 137-146.

Sternlicht, M. D., Sunnarborg, S. W., Kouros-Mehr, H., Yu, Y., Lee, D. C. and Werb, Z. (2005). Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. *Development* **132**, 3923-3933.

Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995). Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.* **270**, 5331-5338.

Sutherland, R. L. and Musgrove, E. A. (2002). Cyclin D1 and mammary carcinoma: new insights from transgenic mouse models. *Breast Cancer Res.* **4**, 14-17.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

Teuliere, J., Faraldo, M. M., Deugnier, M. A., Shtutman, M., Ben-Ze'ev, A., Thiery, J. P. and Glukhova, M. A. (2005). Targeted activation of beta-catenin signaling in basal mammary epithelial cells affects mammary development and leads to hyperplasia. *Development* **132**, 267-277.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442-454.

Vargo-Gogola, T., Fingleton, B., Crawford, H. C. and Matrisian, L. M. (2002). Matrilysin (matrix metalloproteinase-7) selects for apoptosis-resistant mammary cells in vivo. *Cancer Res.* **62**, 5559-5563.

Vincent-Salomon, A. and Thiery, J. P. (2003). Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Res.* **5**, 101-106.

Vu, T. H. and Werb, Z. (2000). Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* **14**, 2123-2133.

Weber, B. H., Vogt, G., Pruett, R. C., Stohr, H. and Felbor, U. (1994). Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby's fundus dystrophy. *Nat. Genet.* **8**, 352-356.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452.

Wilson, C. L., Heppner, K. J., Rudolph, L. A. and Matrisian, L. M. (1995). The metalloproteinase matrilysin is preferentially expressed by epithelial cells in a tissue-restricted pattern in the mouse. *Mol. Biol. Cell* **6**, 851-869.

Wiseman, B. S., Sternlicht, M. D., Lund, L. R., Alexander, C. M., Mott, J., Bissell, M. J., Soloway, P., Itohara, S. and Werb, Z. (2003). Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *J. Cell Biol.* **162**, 1123-1133.

Young, D. A., Phillips, B. W., Lundy, C., Nuttall, R. K., Hogan, A., Schultz, G. A., Leco, K. J., Clark, I. M. and Edwards, D. R. (2002). Identification of an initiator-like element essential for the expression of the tissue inhibitor of metalloproteinases-4 (Timp-4) gene. *Biochem. J.* **364**, 89-99.

Yu, Q., Geng, Y. and Sicinski, P. (2001). Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**, 1017-1021.

Yu, W. H., Yu, S., Meng, Q., Brew, K. and Woessner, J. F., Jr (2000). TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. *J. Biol. Chem.* **275**, 31226-31232.