Breast Cancer Online, **10**(8), Page 1 of 4, ***e16***
doi:10.1017/S1470903107006566

ISSN 1470-9031

Journals Club

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

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Citation of original article:
C. V. Hojilla, I. Kim, Z. Kassiri, J. E. Fat, H. Fang, R. Khokha. ***Journal of Cell Science*** 2007; **120**(6): 1050–1060.

Abstract of the original article:
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.

Review

The Wnt signaling pathway is highly conserved throughout evolution, where it functions in *Caenorhabditis elegans* to mammals [1]. The pathway controls a number of biological responses including cell fate decisions, migration, morphogenesis, differentiation and stem cell renewal. There are three pathways that utilize the 20 known Wnt ligands and 10 frizzled (Fz) family of seven transmembrane receptors [2]. These include the canonical Wnt/β-catenin/Lef pathway where gene transcription is regulated through a transcriptional complex consisting of β-catenin and a member of the Tcf/Lef transcription factor family [3]. Binding of Wnt proteins to the Fz receptor with the co-receptor LRP5 or LRP6 leads to the intracellular activation of

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Received: 24/07/07
Accepted: 30/07/07
BCO/656/2007/JC

Dishevelled, which can then inhibit a second complex of proteins that includes Axin, APC and GSK-3β. In the absence of Wnt signaling, β-catenin is phosphorylated by casein kinase I and GSK-3β in the presence of the scaffolding proteins Axin and APC. This leads to the targeted ubiquitination and degradation of the complex by the proteosome. Activation of Wnt signaling upon ligand stimulation inhibits β-catenin phosphorylation through GSK-3β and thereby stabilizes β-catenin, which leads to its nuclear accumulation and complex formation with Tcf/Lef.

β-Catenin, APC and Axin mutations that promote β-catenin stabilization and prevent degradation are found in a variety of cancers, suggesting that constitutive activation of this pathway leads to tumor formation [4]. In contrast, in breast cancer Wnt mutations are rarely detected. However, overexpression of different members of the Wnt family has been observed [5]. In addition, loss of expression of Wnt inhibitory proteins such as the frizzled-related proteins (sFRPs) or Dickkopf-1, which inhibit Wnt signaling by preventing interaction with Fzs have also been documented in breast tumors [6]. Both effects can lead to the nuclear accumulation of β-catenin and the transcriptional activation of a diverse set of target genes including cyclin D1, c-Myc, MMP3, COX-2, Twist, Snail, Netrin-1, VEGF, HB-EGF and amphiregulin (AR) [7,8]. The two β-catenin-independent Wnt signaling pathways control differentiation, cell shape and cell movements that occur during gastrulation and metastasis. These include the Wnt/Ca^{++} pathway that functions through calcium/calmodulin-dependent protein kinase II and PKC and the Wnt/PCP or planar cell polarity pathway that functions through the GTPases RhoA, Cdc42 and Rac 1, which can then activate Rho-associated kinase (ROCK) and the c-jun kinase (JNK) [9].

Wnt proteins are involved in the early development of the mammary gland during embryogenesis [10]. Lef-1 expression as induced by Wnt 10b in specific regions of the ventral ectoderm leads to the formation of mammary placodes [11]. Postnatal mammary gland development also depends upon the activation of a canonical Wnt/β-catenin/Lef-1 signaling pathway [12]. In the pubescent virgin mammary gland, allometric ductal growth and side-branching are controlled by estrogen and progesterone, respectively. Growth of the mammary tree occurs at the tips of the ducts from the terminal end buds (TEBs). This is an invasive process and specific genes that have been implicated in tumor cell metastasis are activated in the mammary epithelium and/or stroma during this developmental period [13]. In addition, since 90% of breast cancers are of ductal origin, delineating the systemic and local factors that regulate ductal growth may provide important clues to factors that contribute to the early genesis of breast cancer. Branching morphogenesis in the mammary gland was found to depend upon the expression and activation of metalloproteases [14]. For example, MMP3, MMP2 and MMP14 (MT1-MMP), which is an MMP2 activator, are expressed in the mammary stroma, while MMP7 is expressed in the epithelium of the virgin mammary gland. MMP2-null mammary glands are compromized in TEB invasion, whereas MMP3-null mammary glands exhibit reduced side-branching [15]. Overexpression of the MMP inhibitor TIMP1 blocks TEB invasion, suggesting that MMP2 activity is blocked. Overexpression of Wnt1 in the virgin mammary gland that activates a canonical Wnt/β-catenin/Lef-1 signaling pathway can produce precocious alveolar hyperplasia and ductal hyper-branching, which eventually results in the development of mammary adenocarcinomas [16]. An increase in the expression of MMP2, MMP3 and MMP9 occurs in the mammary stroma of Wnt1 transgenic mice whereas MMP13 is expressed by the transformed mammary epithelial cells (MECs), suggesting that additional MMPs other than MMP2 and MMP3 can contribute to tumor progression after sustained activation of a Wnt/β-catenin-dependent pathway [17]. Finally, during pregnancy, lobuloalveolar development is regulated by progesterone and prolactin. Wnt4, which is another canonical Wnt ligand, is upregulated by progesterone during pregnancy and may thus contribute to the enhanced side-branching that is also observed after progesterone treatment [18].

The Wnt signaling pathways can cross-talk with other major signaling pathways such as the canonical TGFβ/Smad pathway and the EGF/ErbB receptor (EGFR) pathway. Transactivation of the EGFR by Wnt1 and Wnt 5a has been demonstrated to occur in HC-11 mouse MECs through the induction of an MMP(s) [19]. Since EGF-like ligands such as TGFα, AR and HB-EGF are initially expressed as cell-tethered transmembrane bound proproteins that can be cleaved by MMPs or by the TACE family of ADAM proteases, this effect of Wnt may account for enhancing EGFR ligand availability. In this context, AR is expressed in the epithelium of the mouse virgin mammary gland and is absolutely required for estrogen-induced ductal growth [20]. In fact, AR is the only EGFR ligand that is required since mice lacking AR but not EGF, TGFα, HB-EGF or betacellulin fail to form ductal outgrowths in the virgin mammary gland [21]. ADAM17 is required for the correct processing of AR in the virgin mammary gland as ADAM17-null mice resemble EGFR-null

https://doi.org/10.1017/S1470903107006566 Published online by Cambridge University Press

and AR-null mice in their phenotype in which the mammary glands fail to develop because of a lack of sufficient ductal growth [22]. Intriguingly, the only endogenous inhibitor of ADAM17 is TIMP3, which is downregulated in the TEBs but is present in the subtending ducts. High levels of AR expression have been found in a large cohort of human breast tumors and in hyperplastic enlarged lobular units (HELUs), which are potential precursors of breast cancer [13,23].

In this paper, Khokha *et al*. demonstrate that TIMP3 can enhance signaling through a β-catenin/Lef pathway in mouse MECs. Previously, this group had shown that MMP3 overexpression in mouse MECs is associated with a decrease in E-cadherin and β-catenin levels. MMP3 and MMP7 can promote invasiveness of MDCK and MCF-7 breast cancer cells by releasing the ectodomain of E-cadherin, thereby reducing adhesion, which is the first step in the process of epithelial-to-mesenchymal transition (EMT). EMT is positively regulated by a number of growth factors including AR, HB-EGF and by activation of the canonical Wnt/β-catenin pathway through the target gene transcription factors Snail and Twist, which can negatively regulate E-cadherin expression. The authors show that loss of TIMP3 alters cell–cell adhesion in a cell type-specific manner. Furthermore, they found that β-catenin signaling was increased and that expression of target genes in this pathway was altered in an MMP- and ADAM-regulated and cell context-specific manner after TIMP3 deletion. TIMP3$^{-/-}$ mouse embryonic fibroblasts (MEFs) from TIMP3-null mice formed much smaller cell aggregates in a time-dependent manner and were more susceptible to detachment by Ca^{++} chelation than wild-type (WT) MEFs, which was independent of any change in E-cadherin or β-catenin expression. In contrast, MECs from TIMP3-null mice were actually more adherent and less susceptible to detachment in the absence of Ca^{++}. There was no change in the localization of either E-cadherin or β-cateinin in the TIMP3$^{-/-}$ MECs. However, there was a significant increase in total E-cadherin and β-catenin in the TIMP3$^{-/-}$ MECs, which was reflected by an increase in nuclear and dephosphorylated (activated) β-catenin. Stimulation of WT and TIMP3$^{-/-}$ MECs with a canonical Wnt ligand, Wnt3A leads to an increase in total β-catenin levels in both cell types with a stronger membrane localization observed in the TIMP3$^{-/-}$ MECs.

To ascertain if there were corresponding changes in any Wnt/β-catenin target genes, the β-catenin/Lef-responsive promoter reporter pTOPFLASH was assessed in this study. The beta-gal activity was ~45% higher in TIMP3$^{-/-}$ MEFs as compared to WT MEFs whereas there was a nearly three-fold increase in promoter activity in the TIMP3$^{-/-}$ MECs as compared to WT MECs. Expression of the canonical Wnt target genes, cyclin D1 or c-Myc was not correlated to any specific genotype in either the MEFs or MECs. However, MMP7 mRNA expression as assessed by qRT-PCR was increased by nearly 10-fold in the TIMP3$^{-/-}$ MECs, demonstrating selective Wnt target gene expression in a cell-context-specific manner after inactivation of TIMP3.

To ascertain whether the increased β-catenin activity after TIMP3 loss was correlated with an increase in MMP or ADAM protease activity, WT MECs were treated with recombinant TIMP3, a metalloprotease inhibitor, GM6001 or with an MMP-specific inhibitor PD166793. TIMP3 treatment lowered the activated form of β-catenin in TIMP3$^{-/-}$ MECs to that of WT MECs. Conversely, treatment of WT MECs with GM6001 increased the levels of active β-catenin to that observed in TIMP3$^{-/-}$ MECs whereas PD166793 decreased the active signaling pool of β-catenin in the TIMP3$^{-/-}$ MECs. These data suggest that Wnt/β-catenin signaling in MECs is influenced by an MMP and/or ADAM activity.

Finally and most importantly, the authors demonstrate the in vivo relevance of these in vitro findings. Virgin mammary glands from WT and TIMP3$^{-/-}$ mice at 3, 4, 6, 9 and 11 weeks were examined for morphological changes by whole-mount analysis. At 3 weeks, the development of the ductal tree was comparable in both genotypes. However, at 4 weeks during puberty the TIMP3-null mammary glands had exhibited accelerated ductal elongation as compared to ductal outgrowth in WT mammary glands. This effect was transient as by 9 and 11 weeks the length of the mammary ducts were equivalent in the two genotypes. The number of ducts or branches per duct was not significantly different at any time point between the two genotypes. Administration of recombinant TIMP3, TIMP1 or TIMP4 in slow-release pellets to WT virgin mammary gland at 3 weeks inhibited ductal elongation whereas TIMP2 actually promoted outgrowth.

The data presented by Khokha *et al*. clearly point to the enormous complexity that exists in the regulation of and the biological activity(ies), which are associated with different TIMPs as these relate to the control of ductal outgrowth and branching morphogenesis in the virgin mammary gland. This complexity is exemplified by the observations that TIMP3 deletion clearly has distinct effects on cell adhesion in fibroblast and MECs, suggesting that MMPs may affect these populations in vivo in quite distinct manners. However, the observation that

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TIMP3 can modulate in a positive fashion canonical Wnt signaling in MECs is intriguing. First, AR and HB-EGF are both canonical Wnt target genes in MECs. In addition, AR expression is also upregulated by estrogen during puberty in mammary ductal epithelial cells through a canonical ER-dependent pathway and adequate expression of AR is essential for normal ductal outgrowth in the virgin mammary gland. Finally, processing of AR and HB-EGF by ADAM17 and MMP7, respectively, can be regulated by upregulation of these genes through a β-catenin-dependent pathway. These data suggest that expression of a subset of EGF-related ligands may be both canonical Wnt gene targets and that processing of these proteins to diffusible paracrine effectors might also be regulated by this pathway. The ability of the negative effector of ADAM17, TIMP3 to upregulate β-catenin-dependent signaling may therefore suggest the activation of a positive feedback loop to compensate for the reduction in AR availability. Loss of expression of TIMP3 may also indicate that it could normally function as a tumor suppressor by preventing the genesis of early-stage breast cancer cells, HELUs.

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