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Wnt signaling activation and mammary gland hyperplasia in MMTV-LRP6 transgenic mice: implication for breast cancer tumorigenesis

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Abstract

Although Wnt signaling activation is frequently observed in human breast cancer, mutations in the genes encoding intracellular components of the Wnt signaling pathway are rare. We found that expression of Wnt signaling co-receptor LRP6 is up-regulated in a subset of human breast cancer tissues and cell lines. To examine whether overexpression of LRP6 in mammary epithelial cells is sufficient to activate Wnt signaling and promote cell proliferation, we generated transgenic mice overexpressing LRP6 in mammary epithelial cells driven by the mouse mammary tumor virus (MMTV) promoter. We found that mammary glands from MMTV-LRP6 mice exhibit significant Wnt activation evidenced by the translocation of β-catenin from membrane to cytoplasmic/nuclear fractions. Expression of several Wnt-target genes including Axin2, Cyclin D1 and c-Myc was also increased in MMTV-LRP6 mice. More importantly, mammary glands from virgin MMTV-LRP6 mice exhibit significant hyperplasia, a precursor to breast cancer, when compared to wild-type littermate controls. Several matrix metalloproteinases are up-regulated in MMTV-LRP6 mice that could contribute to the hyperplasia phenotype. Our results suggest that Wnt signaling activation at the cell surface receptor level can contribute to breast cancer tumorigenesis.

Keywords

LRP6; Wnt signaling; mammary gland; breast cancer

Introduction

The defining feature of the canonical Wnt pathway is the stabilization of cytosolic β-catenin, which enters the nucleus and activates Wnt target genes by binding to transcription factors

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of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family (Giles et al., 2003; Moon et al., 2004). In the absence of Wnt ligands, β-catenin is phosphorylated by a multi-protein complex that marks it for ubiquitination and degradation by the proteasome. This β-catenin degradation complex contains the adenomatous polyposis coli (APC) tumor suppressor, scaffold protein Axin, glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (Ck1). The action of this complex is inhibited upon binding of Wnt to its receptors. Experiments performed in *Drosophila* (Wehrli et al., 2000), *Xenopus* (Tamai et al., 2000) and mice (Pinson et al., 2000) demonstrated that the low- density lipoprotein receptor-related protein 5 (LRP5)/LRP6 (termed *Arrow* in *Drosophila*) acts as a co-receptor for Wnts, which interact with both the seven transmembrane receptor of the Frizzled (Fz) family and LRP5/6 to activate the canonical Wnt signaling pathway.

The role of Wnt/β-catenin signaling in cell proliferation indicates that dysregulation of this pathway may result in cancer. Indeed, several components of the Wnt/β-catenin signaling pathway have been identified as oncogenes or tumor suppressors (showing gain-of-function or loss-of-function mutations, respectively) in human cancers (Giles et al., 2003; Moon et al., 2004). Mutations in these genes are most evident in colorectal cancer. About 85% of all colorectal cancers contain mutations in the tumor suppressor gene *APC*. Mutations in the oncogene encoding β-catenin (*CTNNB1)* are present in approximately 10% of the colorectal cancers. The consequence of either APC inactivation or β-catenin mutation is similar: failure of proper β-catenin degradation leads to its cytosolic accumulation, nuclear translocation, and constitutive activation of β-catenin-responsive genes (Giles et al., 2003; Moon et al., 2004). Although genetic mutations of *APC* or *CTNNB1* are rarely observed in breast cancer, compelling evidence has indicated abnormal regulation of Wnt/β-catenin signaling in breast cancer tumorigenesis (Turashvili et al., 2006; Lindvall et al., 2007). *Wnt1*, the founding member of the *Wnt* genefamily, was initially identified as a mammary oncogene insertionallyactivated by mouse mammary tumor virus (Nusse and Varmus, 1982; Peters et al., 1983; Nusse et al., 1984). Overexpressionof Wnt1, Wnt10b or an activated form of β-catenin *in vivo* results in mammary tumorigenesis (Tsukamoto, et al., 1988; Lane and Leder, 1997), while mice deficient in LRP5 are resistant to Wnt1-induced mammary tumors (Lindvall et al., 2006). Mammary tumors were also observed in heterozygous APC^{Min} mice (Moser et al., 1993). In human breast cancer, secreted Frizzled-related protein1 (sFRP1), a member of the secreted Wnt antagonist family, is down-regulated in malignant tissues (Ugolini et al., 2001; Klopocki et al., 2004). More importantly, β-catenin levels are significantly upregulated and correlate with poor prognosis, acting as a strong and independent prognostic factor in human breast cancer patients (Lin et al., 2000).

LRP6 is expressed in human cancer cell lines and human malignant tissues (Li et al., 2004), and is elevated in testicular germ cell tumors (Rodriguez et al., 2003). Bafico *et al.* reported that there is an autocrine mechanism for constitutive Wnt pathway activation in human cancer cells, and that the autocrine Wnt signaling can be inhibited by siRNA directed against *LRP6* (Bafico et al., 2004). This is the first demonstration that Wnt signaling may be activated in cancerous cells via cell surface Wnt receptors and not due to mutations in one of the downstream signaling components. Our previous studies demonstrated that stable expression of LRP6 in human fibrosarcoma HT1080 cells alters subcellular β-catenin

distribution such that the cytosolic β-catenin level is significantly increased. This is accompanied by a significant increase in Wnt/β-catenin signaling and cell proliferation *in vitro*, and tumor growth *in vivo* (Li et al., 2004). To investigate the role of LRP6 in mammary tumorigenesis, we generated *MMTV-LRP6* transgenic mice and found that overexpression of LRP6 in the mouse mammary gland is sufficient to induce mammary hyperplasia.

Results

Generation of MMTV-LRP6 Transgenic Mice

To assess the potential role of LRP6 in mammary tumorigenesis, we generated a mouse model in which human LRP6 is overexpressed in the mammary gland. The transgenic construct consists of a mouse mammary tumor virus (MMTV) promoter placed upstream of human LRP6 cDNA followed by an SV40 polyadenylation (polyA) site. MMTV-LRP6 mice were generated (named Founder 1–5) and three founders (Founder 1, 4 and 5) carried germline transmission of the LRP6 transgene were identified by RT-PCR (Figure 1a). Real-time quantitative PCR analysis showed that Founder 4 has the highest level of LRP6 transgene expression, whereas Founder 5 has the lowest level (Figure 1b).

To confirm LRP6 expression at the protein level, Western blot analysis of mammary gland lysates prepared from 10-week old MMTV-LRP6 virgin mice was performed. An antibody to detect both endogenous mouse LRP6 as well as the transgenic human LRP6 was used. We found that mammary glands from the transgenic MMTV-LRP6 Founder 4 and 1 displayed 2.6 fold and 1.7 fold greater LRP6 expression levels, respectively, compared to mammary glands from WT littermate controls (Figure 1c). Furthermore, immunohistochemical staining confirmed that LRP6 expression in transgenic MMTV-LRP6 virgin glands was higher than WT virgin glands (Figures 2a, b).

Mammary Gland Hyperplasia in MMTV-LRP6 Transgenic Mice

Whole mount preparations are a well-established method to identify early premalignant lesions of the mammary epithelium (Cardiff et al., 2000). Thus, we performed whole-mount staining of virgin glands to examine the ductal structure of mammary glands in MMTV-LRP6 transgenic mice and WT littermate controls. As shown in Figure 3a, examination of a mammary gland taken from a wild-type littermate control female mouse revealed a branching ductal structure typical of a virgin female. In contrast, inspection of the MMTV-LRP6 mammary gland revealed an unusual number of secondary and tertiary branches and small, spiculated side buds. Quantification of terminal end buds (TEBs) from mammary glands revealed that TEBs from MMTV-LRP6 virgin mice (Founder 4) at 14 weeks (n=4) and 21 weeks (n=4) of age are 3.3 and 6 folds higher, respectively, than those from WT littermate control glands (Figure 3b). Both the founder 1 and 4 lines displayed similar mammary epithelial abnormalities with the founder 4 line having more significant hyperplasia. Furthermore, histological sections of virgin glands showed more individual ducts lined with cuboidal epithelium in transgenic mammary glands than WT littermate control glands (Figures 2c, d). These findings indicate that overexpression of LRP6 in the mouse mammary gland is sufficient to induce mammary gland hyperplasia.

Wnt Signaling Activation in MMTV-LRP6 Transgenic Mice

The ability of LRP6 overexpression to induce mammary gland hyperplasia is likely the result of activation of the Wnt/β-catenin signaling pathway. Transactivation of gene expression by the β-catenin/TCF/LEF complex represents the nuclear target of the Wnt/β-Catenin signaling pathway. To measure TCF/LEF-dependent transcriptional activation we performed reporter gene expression analysis with the Wnt/β-catenin signaling reporter TOPFlash in primary human mammary epithelial cells (HMECs) (Figure 4a). A vector with mutated copies of the TCF/LEF (FOPFlash) was used for measuring non-specific trans-activation (Figure 4b). We found that transient transfection of LRP6 into HMECs results in a significant increase of TOPFlash luciferase activity, an effect blocked by cotransfection with Dkk1 (Figure 4a). In addition, Wnt3A treatment greatly enhanced the effect of LRP6 on TOPFlash luciferase activity in HMECs (Figure 4a).

Uncomplexed cytosolic β-catenin (free β-catenin) is the active form of β-catenin that is translocated to the cell nucleus, which activates transcription factors of the TCF/LEF family, leading to the transcription of Wnt target genes (Bafico et al., 1998 & 2004). We then examined the extent of Wnt/β-catenin signaling in mammary glands from MMTV-LRP6 mice or WT littermate controls by determining the levels of free β-catenin present in the cytoplasma of mammary tissues. While there was no significant difference of total cellular β-catenin in mammary glands between MMTV-LRP6 transgenic mice and WT littermate controls, virgin glands from MMTV-LRP6 transgenic mice (founder 4) exhibited higher levels of cytoplasmic free β-catenin than WT littermate controls Figure 5a). Quantification of Western blot signals revealed that expression levels of cytoplasmic free β-catenin in mammary glands from MMTV-LRP6 transgenic mice are 1.9 folds higher than those from WT littermate controls. Furthermore, we prepared mammary gland nuclear extracts and found that the levels of both total β-catenin and unphosphorylated, active β-catenin in mammary nuclear extracts from MMTV-LRP6 transgenic mice were higher than those from WT littermate controls (Figures 5b, c). All together, these data suggest that increased LRP6 expression in mammary glands promotes Wnt/β-catenin signaling by altering β-catenin subcellular distribution.

Axin2 (Yan et al., 2001; Jho et al., 2002; Leung et al., 2002; Lustig et al., 2002) is a direct and specific transcriptional target of the Wnt/β-catenin signaling pathway. It is well recognized that the expression level of Axin2 is the signatureof the activation of Wnt/β-catenin signaling. To further confirm that Wnt/β-catenin signaling is up-regulated in MMTV-LRP6 transgenic mammary glands, we examined the expression of Axin2 by q-PCR. As expected, Axin2 in mammary glands from MMTV-LRP6 transgenic mice were 3.3 folds higher than those from WT littermate controls (Figure 6a).

c-Myc and Cyclin D1 are key transcriptional targets of the Wnt/β-catenin pathway (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). To identify a potential mechanism by which LRP6 accelerates the development of dysplastic mammary lesions, we examined the expression of c-Myc and Cyclin D1 in mammary tissues. As expected, virgin glands from MMTV-LRP6 transgenic mice exhibited higher levels of c-Myc and Cyclin D1 expression than WT littermate controls (Figure 6b). Quantification of the Western blot signals revealed that expression levels of c-Myc and Cyclin D1 in mammary glands from

MMTV-LRP6 transgenic mice are 1.5 and 3.3 folds higher, respectively, than those from WT littermate controls (Figure 6c).

C-Myc and Cyclin D1 are two important cell cycle regulators. Having established that the expression levels of c-Myc and Cyclin D1 in mammary glands from MMTV-LRP6 transgenic mice are up-regulated, we then examined the proliferation status of mammary epithelial cells. Ki67 is a nuclear protein that is tightly linked to the cell cycle, and a marker of cell proliferation. Indeed, immunohistochemical staining revealed that Ki67 expression in transgenic MMTV-LRP6 virgin glands was higher than WT virgin glands (Figures 6d, e). In contrast, TUNEL staining revealed that there was no significant difference in apoptosis status between transgenic MMTV-LRP6 virgin glands and WT virgin glands (Supplement Figure 1), suggesting that the regulation of apoptosis is not involved in mammary gland hyperplasia of MMTV-LRP6 mice.

MMP Expression in MMTV-LRP6 Mammary Glands

The matrix metalloproteinases (MMPs) are known to degrade extracellular matrix proteins and to perform key functions in tissue development and tumor progression (Egeblad and Werb, 2002). MMP-2, -3, -7, -9, -13 and -14 are all known target genes of the Wnt/β-catenin pathway (Crawford et al., 1999; Takahashi M et al., 2002; Tamamura et al., 2005; Wu, et al., 2007). To further identify potential mechanisms by which LRP6 accelerates the development of dysplastic mammary lesions, we performed real-time quantitative PCR analysis to detect expression levels of MMP-2, -3, -7, -9, -13 and -14 expression in virgin glands from 12-week old mice. As shown in Figure 7, MMP levels in mammary glands from MMTV-LRP6 transgenic mice were 2–10 folds higher than those from WT littermate controls.

Discussion

While genetic mutations of certain intracellular components of the Wnt/β-catenin pathway, such as *APC* and *CTNNB1*, are significant contributing factors for colorectal cancers, they are typically not the predominate mechanism associated with other cancer types such as breast cancer. Instead, it appears that dysregulation of cell surface Wnt/β-catenin signaling components leads to aberrant activation of this pathway in breast cancer (Turashvili et al., 2006; Lindvall et al., 2007). We found that expression of the Wnt signaling co-receptor LRP6 is up-regulated in a subset of human breast cancer tissues and cell lines (unpublished data). In the present study, we demonstrated that transgenic mice over-expressing LRP6 in mammary epithelial cells driven by the MMTV promoter is sufficient to induce mammary gland hyperplasia, a precursor to breast cancer. During the revision process of the manuscript, Lindvall et al. reported that canonical Wnt signaling through LRP6 is required for normal mouse mammary gland development, and that LRP6 expression is increased in basal-like human breast cancer, a triple-negative phenotype associated with high grade, poor prognosis, and younger patient age (Lindvall et al., 2009). Altogether, these findings indicate that mammary tumorigenesis can be initiated at the cell surface receptor level in mammary epithelium, and that LRP6 is a potential target for breast cancer therapy.

Cyclin D1 and c-Myc are two important cell cycle regulators. Clinically, the *Cyclin D1* gene is amplified in up to 20% of human breast cancers and Cyclin D1 protein is overexpressed in >50% of human mammary carcinomas (Bartkova et al., 1994; Gillett et al., 1994; McIntosh et al., 1995). For c-Myc, a comprehensive meta-analysis suggests that at least 15% of breast cancers present with significant amplification of *c-Myc*, and that *c-Myc* amplification is significantly associated with a poor prognosis in breast cancer (Deming et al., 2000). In animal studies, transgenic mice over-expressing Cyclin D1 in mammary epithelium (MMTV-Cyclin D1) develop mammary hyperplasia and mammary carcinomas (Wang et al., 1994). Similarly, constitutive c-Myc expression under the control of MMTV or the whey acidic protein promoters is oncogenic in transgenic mice (reviewed in Amundadottir et al., 1996; Nass and Dickson, 1997). Amplification of *c-Myc* or *Cyclin D1* has been identified as a downstream step at the end of Wnt/β-catenin pathway activation (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Therefore, it is expected that LRP6 overexpression would cause the activation of Wnt/β-catenin signaling, up-regulation of Cyclin D1 and c-Myc expression levels, and increases the expression of the cell proliferation marker Ki67, all of which we observed experimentally in MMTV-LRP6 mice. As such, these events could account for mammary hyperplasia in MMTV-LRP6 mice.

MMPs are multifunctional enzymes capable of targeting the extracellular matrix, growth factors, cytokines and cell surface-associated adhesion and signaling receptors. Clinically, MMPs have been associated with advanced-stage cancer and contribute to tumor progression, invasion, and metastasis. In ductal breast carcinomas, it has been demonstrated that MMP-2, -3, -9, -11, -13 and -14 are synthesized either by stromal fibroblasts, infiltrating macrophages or vascular pericytes (Wolf et al., 1993; Okada et al., 1995; Heppner et al., 1996; Nielsen et al., 1997 & 2001; Chenard et al., 1999). In animal models, it has been found that expression of an autoactivating form of MMP-3 under the control of the whey acidic protein promoter induces premalignant and malignant lesions in the mammary glands. Moreover overexpression of a natural inhibitor of MMPs, tissue inhibitor of MMP (TIMP)-1, inhibits tumor formation in MMP-3 transgenic mice (Sternlicht et al., 1999). More interestingly, Blavier et al. recently reported that the expression of several MMPs including MMP-2, -3, -9,-13, and -14 was increased in hyperplastic glands and mammary tumors of MMTV-Wnt1 transgenic mice. Furthermore, when MMTV-Wnt1 mice were crossed with transgenic mice overexpressing a natural MMP inhibitor, TIMP2, in the mammary gland, the double transgenic mice displayed an increase in tumor latency and a reduction in tumor formation (Blavier et al., 2006). MMP-2, -3, -7, -9, -13 and -14 are all known target genes of the Wnt/β-catenin pathway (Crawford et al., 1999; Takahashi et al., 2002; Tamamura et al., 2005; Wu et al., 2007). In the present study, we demonstrated that mammary glands from MMTV-LRP6 transgenic mice exhibit significantly higher levels of MMPs than WT littermate controls. Therefore, upregulation of MMPs could also account for mammary hyperplasia in MMTV-LRP6 mice.

Whole mount staining of mammary gland is a well-established and recommended method to identify early premalignant lesions of the mammary epithelium (Cardiff et al., 2000). In our mammary gland model, we found that the MMTV-LRP6 female mice exhibit mammary gland hyperplasia. However, none of the MMTV-LRP6 mice developed adenocarcinoma of

breast for more than an 18-month span. Therefore, it is possible that the extent of the Wnt/β-catenin activation upon LRP6 overexpression is sufficient to cause mammary gland hyperplasia but not sufficient to lead to breast cancer in virgin mice. It will be interesting to examine whether multiparous MMTV-LRP6 mice develop breast cancer. Crossing MMTV-LRP6 mice with MMTV-Wnt mice will allow us to test whether LRP6 and Wnt1 synergistically promote breast cancer tumorigenesis. Together, these studies should help to define whether LRP6 and Wnt ligands are novel targets for breast cancer therapy.

Material and methods

Generation of MMTV-LRP6 mice

To generate the MMTV-hLRP6 construct, human LRP6 cDNA fragment was removed from the pCS-myc-hLRP6 vector (kindly provided by Dr. Christof Niehrs, German Cancer Research Center) by digestion with *Cla* and *XbaI*, followed by *n*-fill with Klenow enzyme. The LRP6 fragment was then blunt-end ligated into the *EcoRI* site (*n*-filled) of the MMTV-SV40-Bssk vector (kindly provided by Dr. Philip Leder, Harvard Medical School). Purified MMTV–LRP6 DNA fragment was microinjected into the pronuclei of fertilized mouse eggs, and transferred to pseudopregnant mothers by the Transgenic and ES Cell Core of Washington University School of Medicine. Mice were genotyped for the presence of the transgenes by PCR with primers 5′-GGCTATACCAGTGACTTGAACTATGATT-3′, and 5′-GGTTCCTTCACAAGATCCTCTAGAGTC-3′ to identify the MMTV–LRP6 transgene.

RNA isolation and quantitative PCR

Total RNA from inguinal mammary gland (number 4) of 14-week old mice was extracted by SV total RNA isolation kit (Promega, Z3100). Total RNA was dissolved in nuclease-free water and stored at −80°C. Reverse transcription was performed using a SuperScript II RNase H-reverse transcriptase (Invitrogen), and the reaction mixture was subjected to either reverse transcription-PCR (RT-PCR) or quantitative real-time PCR (q-PCR) to detect levels of human LRP6, MMPs, Axin2 and actin, which was used as an internal control. For a 50 μl of q-PCR reaction, 25 μl of SYBR Supermix from BioRad, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 2 μl of mixture of reverse transcription reaction, and 21 μl of water were included. After 40 cycles, the relative levels of gene expression were quantified with Bio-Rad iCycler iQ software. The primers used to amplify target genes by RT-PCR and q-PCR were as following: hLRP6-F (5′ TAGCATTGAAAGAGTTCATAAACGA 3′), hLRP6-R (5′ CACTCGATGAACATTTGTAGCCTTT 3′); Axin2-F (5′ TGACTCTCCTTCCAGATCCCA 3′), Axin2-R (5′ TGCCCACACTAGGCTGACA 3′); MMP2-F (5′ CAAGTTCCCCGGCGATGTC 3′), MMP2-R (5′ TTCTGGTCAAGGTCACCTGTC 3′); MMP3-F (5′ TGTCCCGTTTCCATCTCTCTC 3′), MMP3-R (5′ TGGTGATGTCTCAGGTTCCAG 3′); MMP7-F (5′ CTGCCACTGTCCCAGGAAG 3′), MMP7-R (5′ GGGAGAGTTTTCCAGTCATGG 3′); MMP9-F (5′ GGACCCGAAGCGGACATTG 3′), MMP9-R (5′ CGTCGTCGAAATGGGCATCT 3′); MMP13-F (5′ ACCTCCACAGTTGACAGGCT 3′), MMP13-R (5′ AGGCACTCCACATCTTGGTTT 3′); MMP14-F (5′ CAGTATGGCTACCTACCTCCA G 3′), MMP14-R (5′ GCCTTGCCTGTCACTTGTAAA 3′).

Western blot analysis

Thoracic and inguinal mammary glands from both wild type and transgenic mice were lysed with phosphate-buffered saline containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and the protease inhibitor cocktail from Roche. Protein concentrations were determined with Protein Assay kit from Bio-Rad. Equal amount of proteins from each sample was used for SDS–PAGE. The immunoreactive bands were visualized by enhanced chemiluminescence and exposure to film. For densitometric analyses, immunoreactive bands were scanned using a Kodak Digital Science DC120 Zoom camera and quantified using Kodak Digital Science image analysis software. Human LRP6 antibody was from R&D. c-Myc and Cyclin D1 antibodies were from Santa Cruz. β-catenin antibody was from BD Transduction Laboratories, active β-catenin antibody was from Millipore. β-Actin antibody was from Sigma.

Luciferase reporter assay

Wnt3A conditioned medium (Wnt3A CM) and L cell control CM were prepared as previously described (Lu et al., 2008). Normal human mammary epithelial cells (HMECs) were purchased from Lonza. HMECs were plated into 24-well plates. For each well, 0.05 μg of the TOPFlash TCF luciferase construct (Upstate Biotechnology) or the negative control FOPFlash TCF luciferase construct was co-transfected with LRP6-, Dkk1-expressing vector, or empty pcDNA3 vector. A β-galactosidase-expressing vector (Promega, Madison, WI) was included as an internal control for transfection efficiency. After 24 h incubation, the cells were treated with 5% of Wnt3A CM or L cell control CM. After further 24 h incubation, cells were lysed and both luciferase and β-galactosidase activities were determined with enzyme assay kits (Promega). Luciferase activity was normalized to the activity of the β-galactosidase.

GST-E-cadherin binding assay for cytoplasmic free β-catenin

The GST-E-cadherin binding assay was carried out as previously described (Lu et al., 2008). Briefly, recombinant GST-E-Cadherin protein was expressed, purified and conjugated with agarose beads. The GST E-cadherin beads were incubated with 100 μg of total cell lysates prepared from mammary gland tissues of MMTV-LRP6 mice and WT littermate controls for 4 h at 4°C. This process allows uncomplexed cytoplasmic freeβ-catenin in total cell lysates to bind to GST E-Cadherin beads. The cytoplasmic free β-catenin was then eluted from the GST E-Cadherin beads, subjected to SDS-PAGE, and detected using a monoclonal antibody to β-catenin.

Preparation of crude membrane fractions

Membrane fractionation can enrich membrane proteins to be detected. Thoracic and inguinal mammary glands from both MMTV-LRP6 transgenic mice and WT littermate controls were harvested and immediately put into prechilled 20 mM Tris pH 8.0 containing 150 mM NaCl, 1 mM $CaCl_2$ and EDTA-free Complete proteinase inhibitors. After homogenization with a dounce homogenizer and low-speed centrifugation at 1500g for 5 mins, a small portion of homogenate was kept at 4°C as whole tissue lysate, while the majority of homogenate was further centrifuged at 100,000g for 30 min at 4°C to separate the membrane fraction

(insoluble pellet) from cytoplasmic fraction (supernatant). The insoluble pellet was resuspended in 50 mM Tris pH 8.0 containing 80 mM NaCl, 2 mM $CaCl_2$ and EDTA-free complete proteinase inhibitors. Triton X-100 was then added to whole tissue lysate, membrane and cytoplasmic fractions at 1% final concentration. The membrane fraction was passed several times through a 28-gauge needle and cleared further by centrifugation at 100,000g for 15 min. Total protein concentrations were determined with Protein Assay kit (Bio-Rad). Equal amounts of total protein were separated by SDS-PAGE under reducing condition, and analyzed by Western Blotting. To validate the membrane protein preparation, Western blot analysis was performed with antibodies against cell surface receptor LRP1 (Bu et al., 1995) and nuclear marker Lamin B1 (Abcam) (Supplement Figure 2a).

Nuclear extraction

NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific) was used for preparation of nuclear and cytoplasmic fractions from mammary gland tissues. To validate the purity of the nuclear and cytoplasmic fractions, Western blot analysis was performed with antibodies against nuclear marker Lamin B1 (Abcam) and cytoplasmic marker HSP90 (Cell Signaling Technology) (Supplement Figure 2b).

Whole mount staining of mammary gland

Inguinal mammary glands (number 4) were dissected at the indicated times of development and spread on glass slides. After fixation in Carnoy's fixative for 4 h at room temperature, the tissues were hydrated and stained in carmine alum as described on http://mammary.nih.gov. Samples were then dehydrated, cleared in Histoclear, and photographed. Counting of terminal end buds (TEBs) was performed by defining counting areas first, drawing 1mmX1mm squares on whole mount pictures, and taking the average number of TEBs from 5 randomly picked areas.

Immunohistochemistry

Inguinal mammary glands (number 4) were dissected from both MMTV-LRP6 transgenic mice and WT littermate controls, fixed in 4% phosphate-buffered paraformadehyde overnight, transferred to 70% ethanol, embedded in paraffin, and sectioned at 5μm. After deparaffinization, rehydration and antigen retrieval by heating in antigen unmasking solution (Dako, Carpinteria, CA), tissue slices were blocked with serum using Vectastain ABC kit (Vector Laboratories, Burlingame, CA), incubated with primary anti-hLRP6 antibody (R&D, Indianapolis, IN) or Ki67 antibody (Abcam, Cambridge, CA) at 4°C overnight. After washing with PBS, the slices were incubated with biotinylated secondary antibody for 30 min at room temperature, and detected by Histostain Plus DAB kit (Zymed Laboratories, CA).

H & E staining

Inguinal mammary glands (number 4) were excised, fixed with paraformadehyde, and embedded in paraffin. Sections were cut at 5 μm, dewaxed, rehydrated, and stained with hematoxylin and eosin.

TUNEL staining

Paraffin sections at 5 μm of mammary glands from MMVT-LRP6 and WT littermate controls were depariffinized. TUNEL staining was performed with TUNEL Apoptosis Detection kit (Upstate) following manufacturer's instructions, and nuclei were counterstained with DAPI 5 μg/ml for 5 min.

Statistical Analysis

All quantified data represent an average of at least three samples. Error bars represent standard deviation. Statistical significance was determined by Student's test, and $p<0.05$ was considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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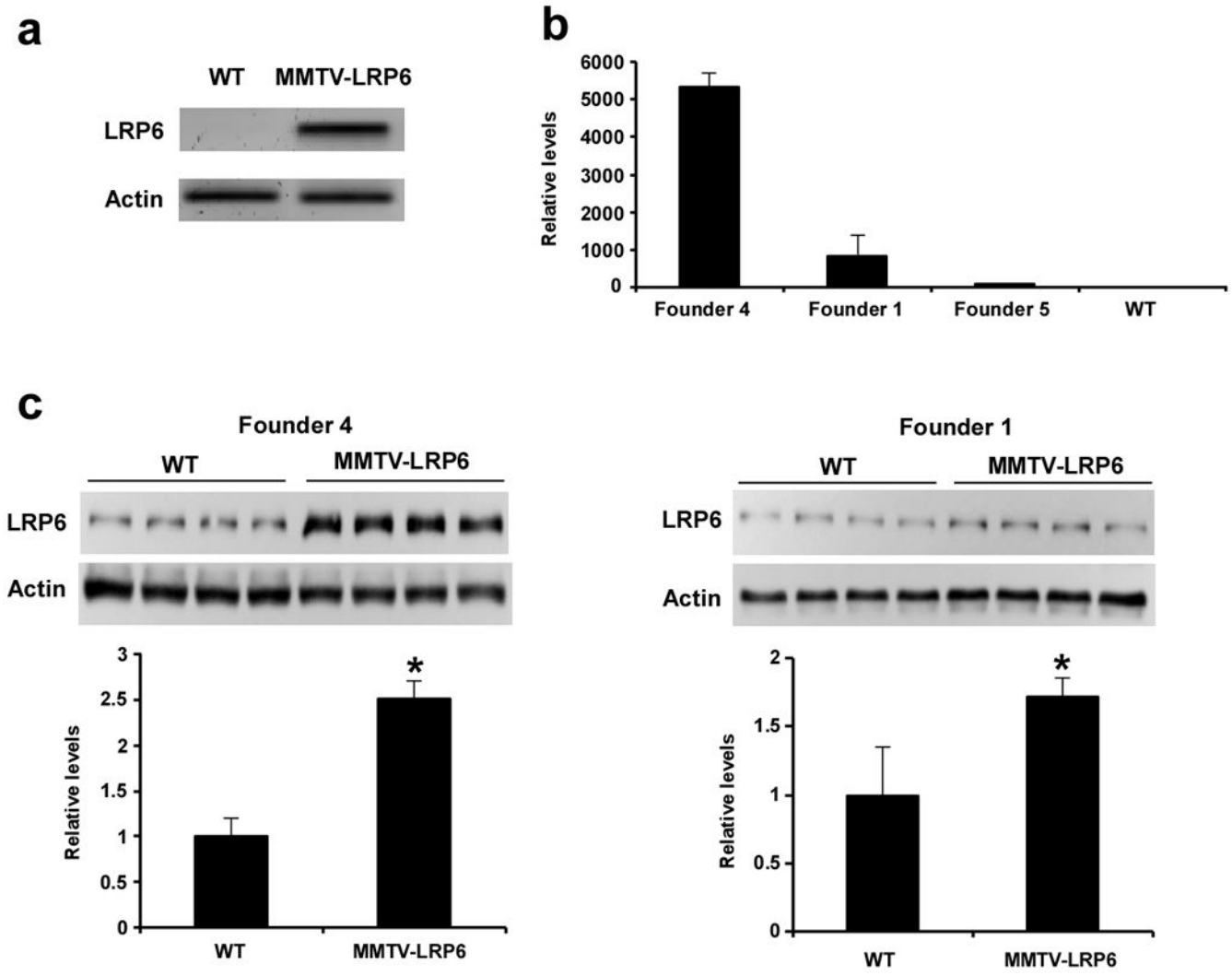

Figure 1.

Generation and characterization of MMTV-LRP6 mice. (a) RNA isolated from mammary glands of MMTV-LRP6 virgin mice and WT littermate controls (12 weeks old) was used for RT-PCR to detect myc-tagged human LRP6 transgene in Founder 4. (b) Quantification of LRP6 transgene expression by q-PCR. Founder 4 has the highest level of LRP6 transgene expression, while Founder 5 has the lowest. (c) Mammary glands from MMTV-LRP6 mice or WT littermate controls (12 weeks old, n=4) were dissected and lysates were prepared. The levels of LRP6 in crude membrane fraction were analyzed by Western blotting with a specific LRP6 antibody. Samples were also probed with an anti-actin antibody to verify equal loading. Lower panel, quantification of the Western blot signals of LRP6 from mammary glands, which were normalized to actin levels. Error bars represent SD. *P<0.05 indicates a significant difference compared to mammary glands from WT littermate controls.

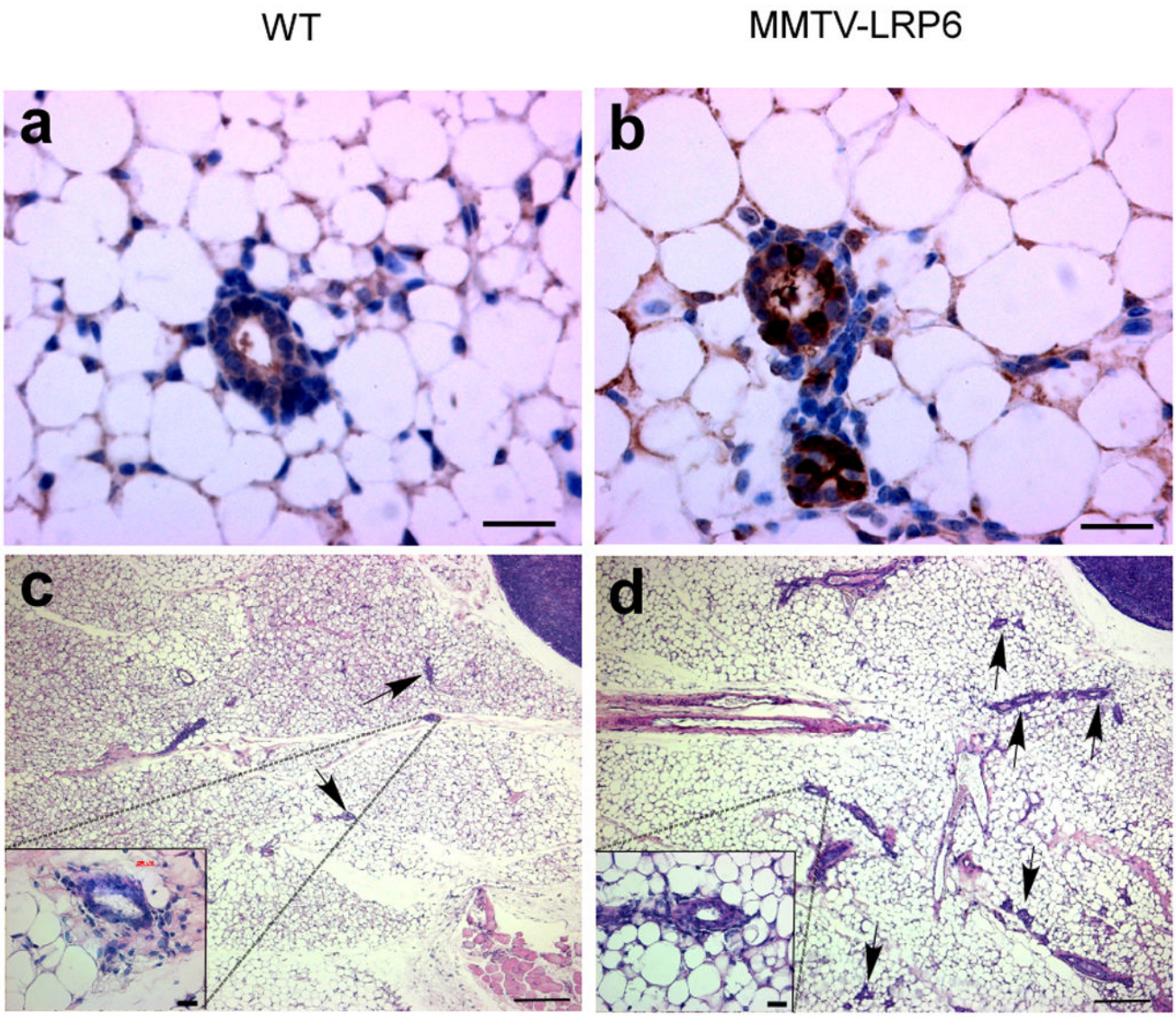

Figure 2.

LRP6 immunohistochemical staining in the mammary glands of MMTV-LRP6 mice. (a–b) LRP6 expression in mammary glands of MMTV-LRP6 virgin mice and WT littermate controls was shown through immunohistochemical staining using human LRP6 polyclonal antibody. Bar, 200 μm. (c–d) H & E staining of mammary glands from MMTV-LRP6 virgin mice and WT littermate controls. Bar, 400 μm, inset, 200 μm.

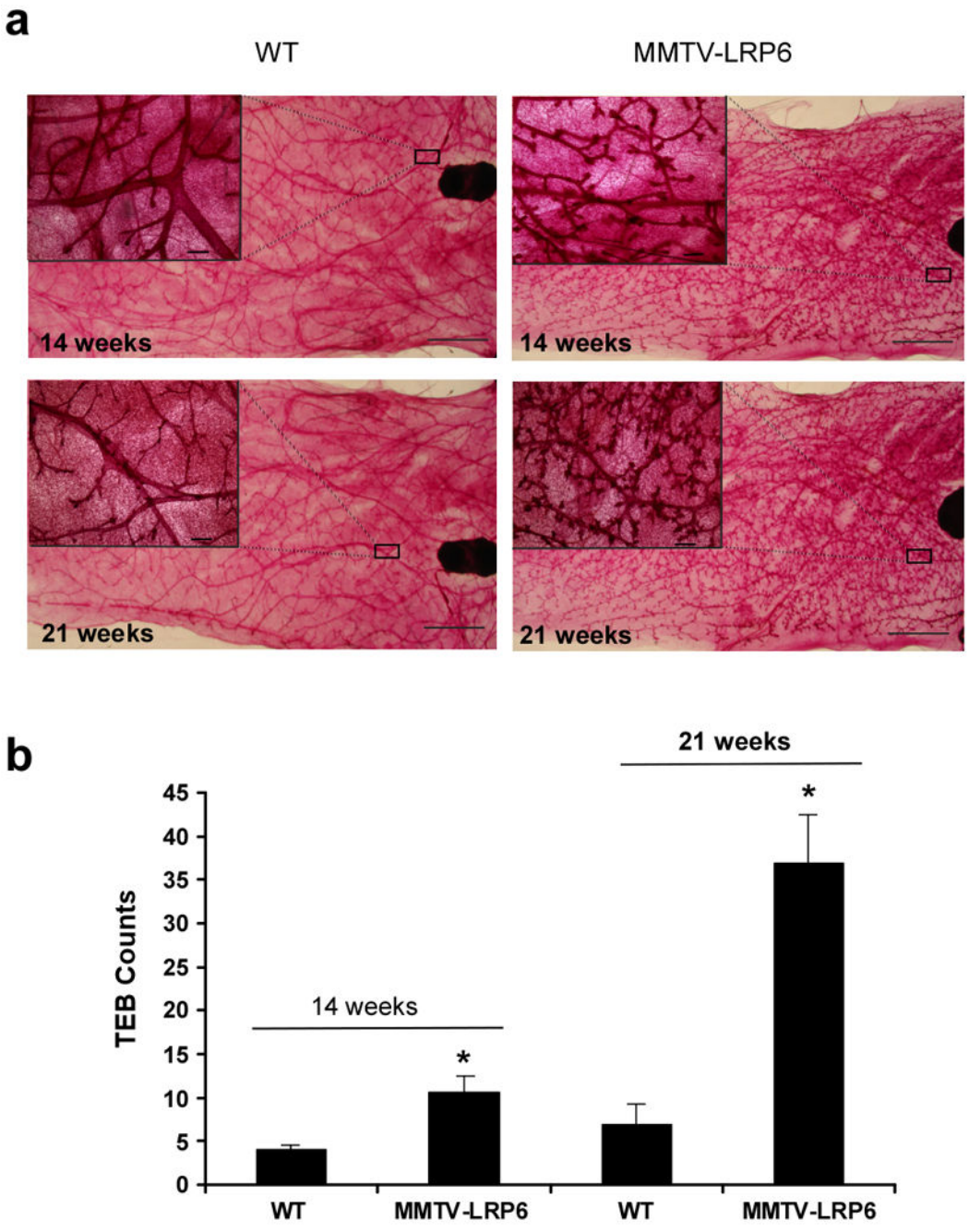

Figure 3.

Mammary gland hyperplasia in MMTV-LRP6 mice. (a) Mammary glands from MMTV-LRP6 virgin mice or WT littermate controls (Founder 4 line) at 14 or 21 weeks of age were analyzed by whole mount staining. *Inset*: high magnification of a selected area. Note mammary hyperplasia in MMTV-LRP6 mice. Bar, 500 μm, inset, 400 μm. (b) Quantification of terminal end buds (TEBs) from WT and MMTV-LRP6 mice (Founder 4, n=4) at 14 or 21 weeks of age. Error bars represent SD. *P<0.05 indicates a significant difference compared to mammary glands from WT littermate controls.

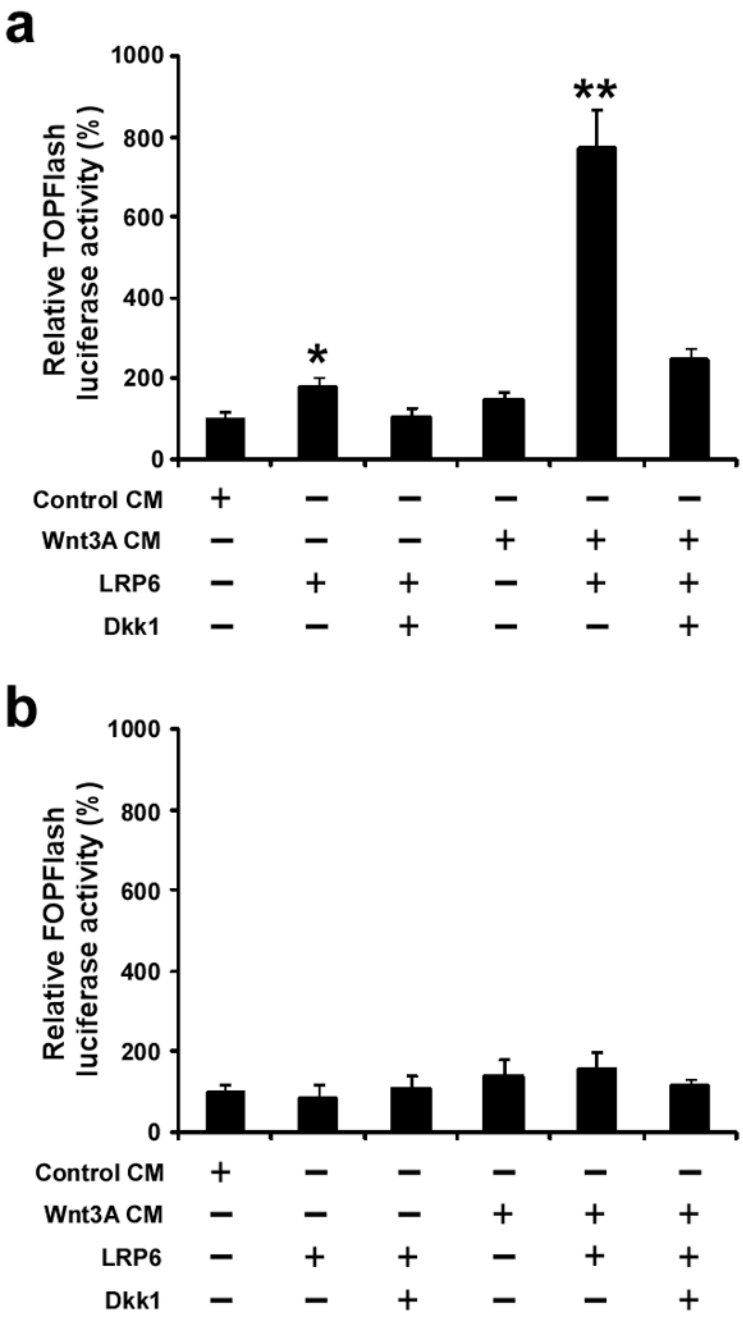

Figure 4.

Wnt signaling is up-regulated in HMECs by LRP6 overexpression. HMECs were cultured in 24-well plates. For each well, 0.05 μg of Wnt signaling reporter construct TOPFlash (a) or FOPFlash (b), and 0.05 μg of the β-galactosidase-expressing vector were co-transfected with 0.05 μg of LRP6-, Dkk1-expressing vector, or empty pcDNA3 vector. After 24 h, cells were treated with 5% of Wnt3A CM or L cell control CM. After further 24 h incubation, cells were lysed and both luciferase and β-galactosidase activities were determined. The luciferase activity was normalized to the activity of the β-galactosidase. All values are the average of triple determinations with the SD indicated by error bar. *P<0.05 indicates a

significant difference compared to HMECs transfected with LRP6 and Dkk1, or with empty vector only; **P<0.01 indicates a significant difference compared to HMECs transfected with LRP6 or Dkk1 only, or to HMECs transfected with LRP6 and Dkk1 and treated with Wnt3A CM.

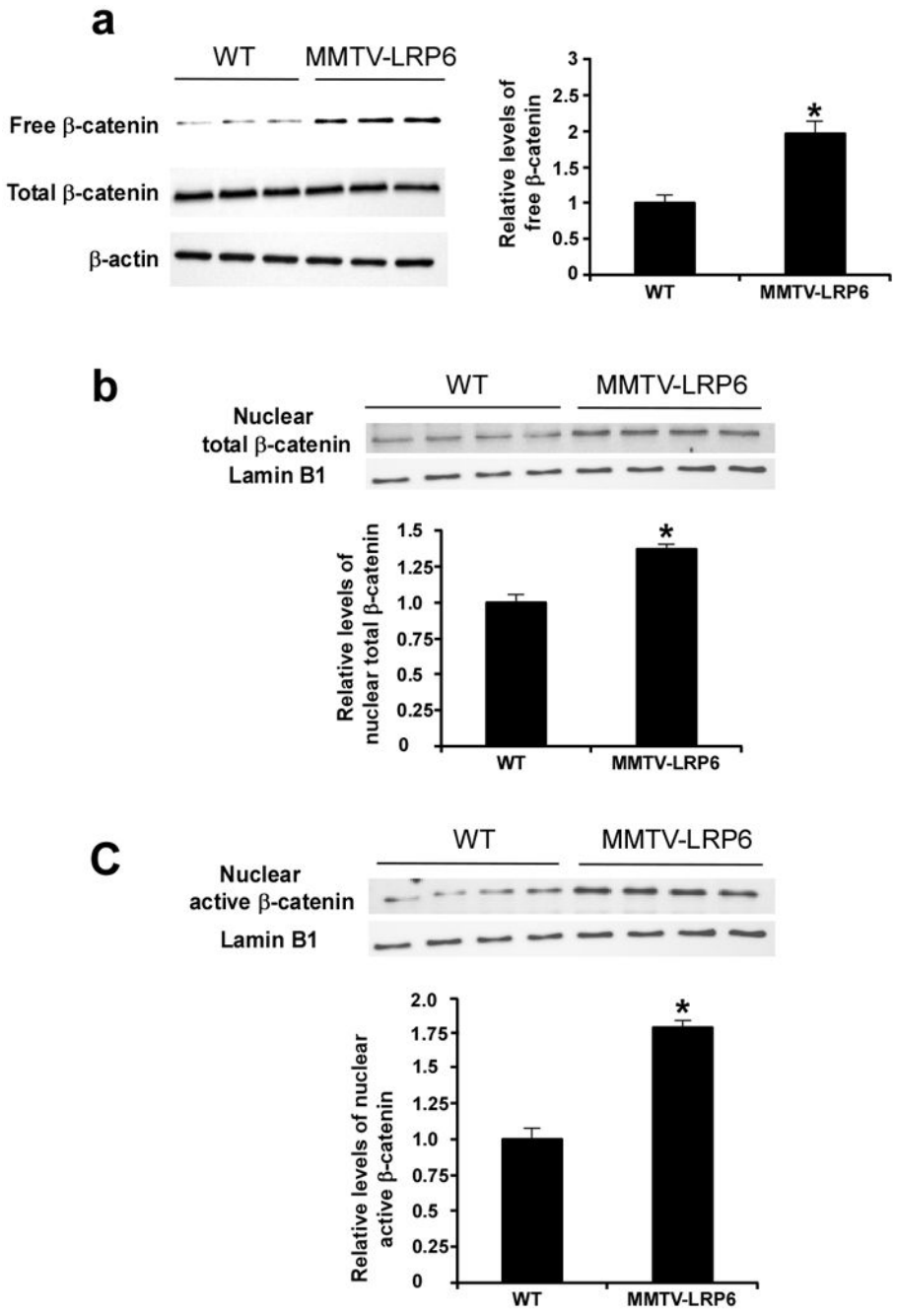

Figure 5.

Wnt/β-catenin signaling activation in the mammary glands of MMTV-LRP6 mice. (a) Virgin glands from MMTV-LRP6 mice or WT littermate controls (14 weeks old, n=3) were dissected and lysates were prepared. Cytoplasmic free β-catenin was pulled down from 200 μg of cell lysate using pGST-E-Cadherin, and then examined by Western blotting with a specific β-catenin antibody. The levels of total cellular β-catenin and actin were also analyzed by Western blotting. Right panel, quantification of the Western blotting signals of cytoplasmic free β-catenin. (b–c) Nuclear extracts from mammary glands of MMTV-LRP6 mice or WT littermate controls (14 weeks old, n=3) were prepared by fractionation, and

examined by Western Blotting with either regular β-catenin antibody (b) or activeβ-catenin (unphosphorated β-catenin) antibody (c). Lamin B1 was blotted as a loading control. Lower panel, quantification of the Western blot signals of β-catenin from mammary gland nuclear extracts. The levels of β-catenin were normalized to the Lamin B1 levels. Error bars represent SD. *P<0.05 indicates a significant difference compared to mammary glands from WT littermate controls.

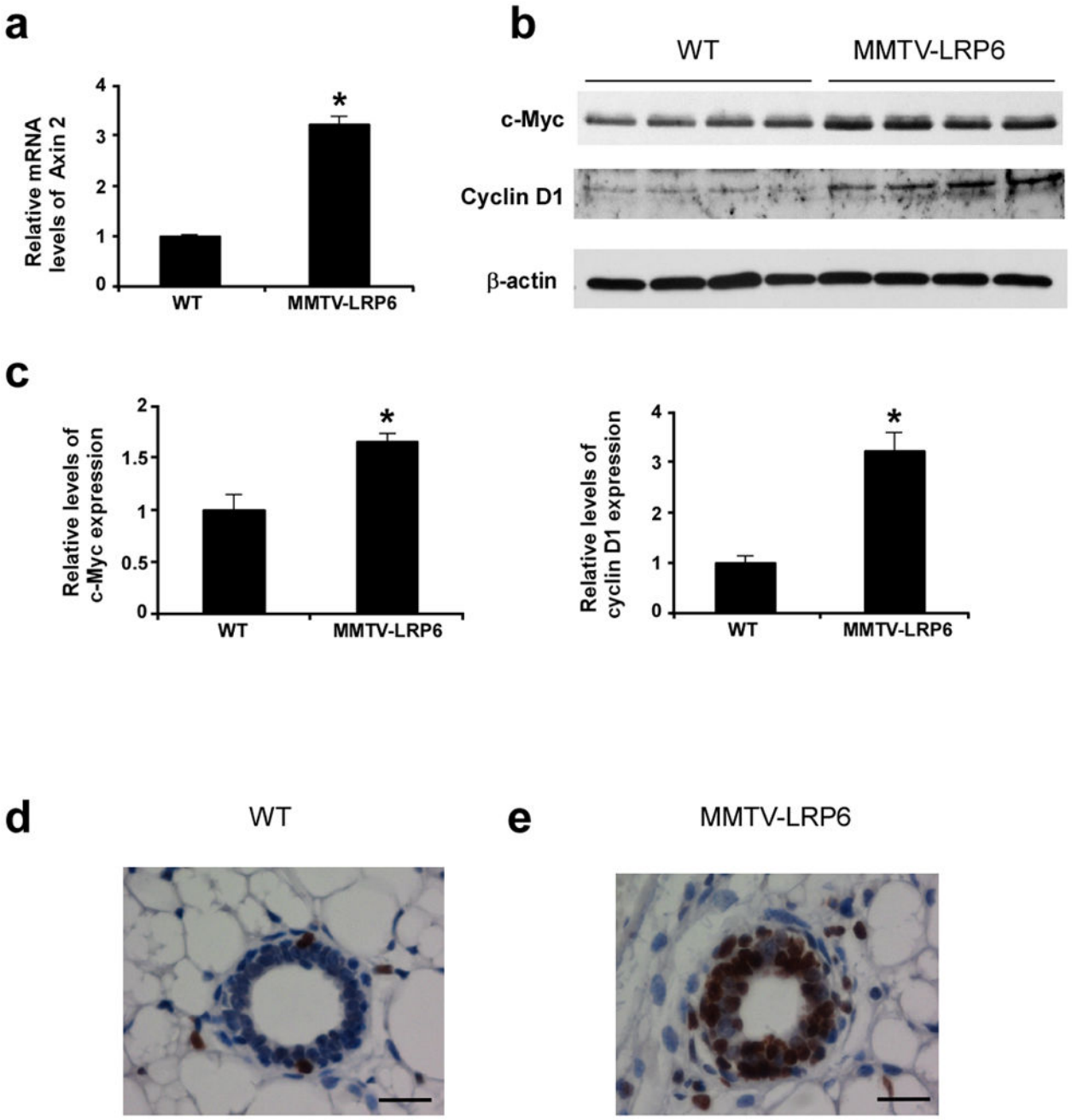

Figure 6.

Axin2, c-Myc, Cyclin D1 and Ki-67 are up-regulated in MMTV-LRP6 mice. (a) Total RNA was isolated from virgin mammary gland of MMTV-LRP6 virgin mice and WT littermate controls (4 weeks, n=3). The levels of Axin2 were measured by q-PCR. Fold changes in expression compared to WT littermate controls were plotted. (b) Virgin glands from MMTV-LRP6 mice or WT littermate controls (14 weeks old, n=4) were dissected, and lysates were prepared. The levels of total cellular c-Myc and Cyclin D1 were analyzed by Western blotting with a specific c-Myc antibody or Cyclin D1 antibody, and the samples were also probed with an anti-actin antibody to verify equal loading. (c) Quantification of

the Western blot signals of c-Myc and Cyclin D1 expression described in (b). The levels of c-Myc and Cyclin D1 expression were normalized to the actin levels. (d, e) Ki67 immunostaining of mammary glands from MMTV-LRP6 virgin mice (e) and WT littermate controls (d). Bar, 200 μm. Error bars represent SD. *P<0.05 indicates a significant difference compared to mammary glands from WT littermate controls.

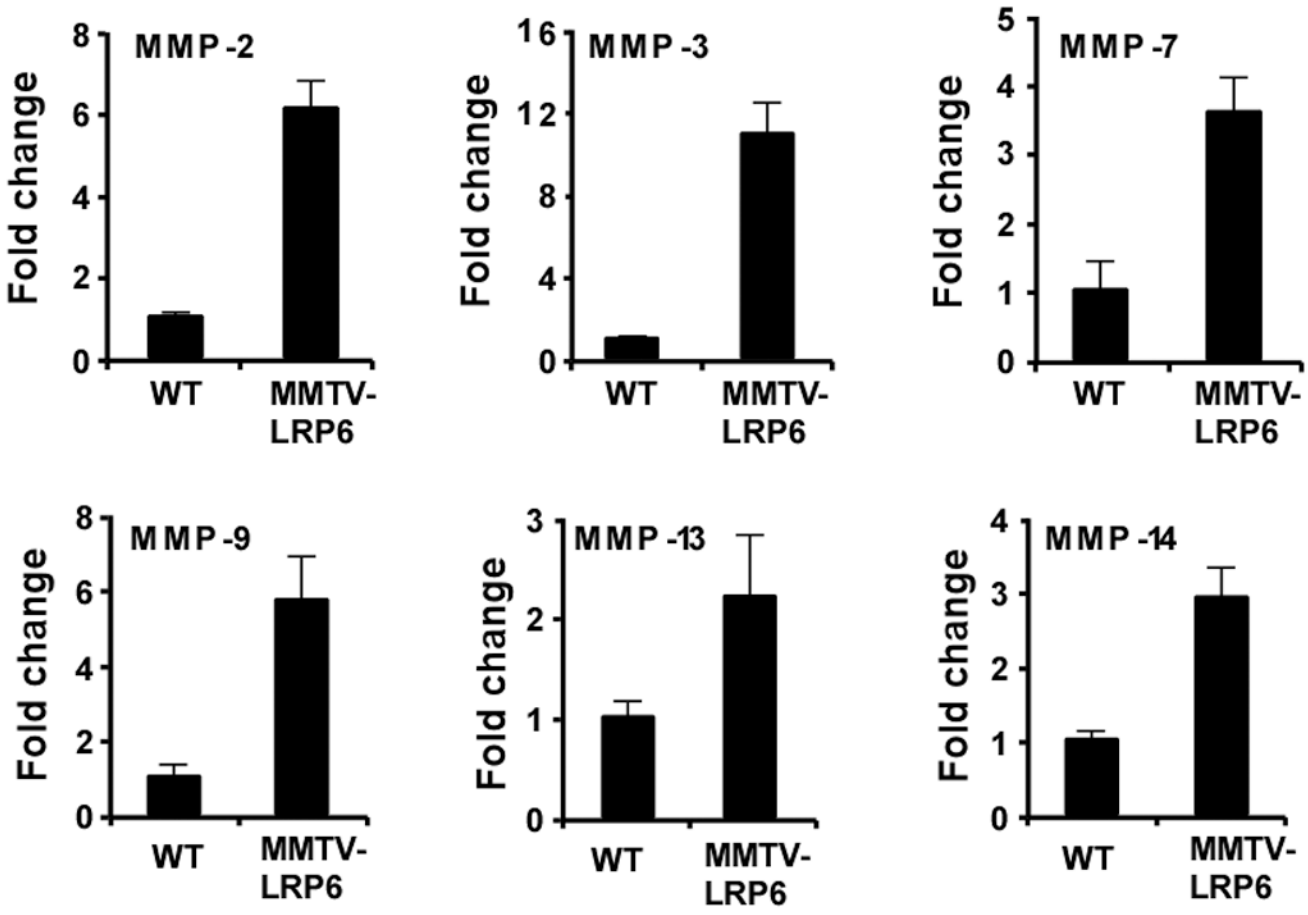

Figure 7.

MMPs are up-regulated in MMTV-LRP6 mice. RNA from mammary glands of 14-month old MMTV-LRP6 virgin mice and WT littermate controls were prepared, and the expression levels of 6 MMPs were measured by q-PCR. Fold changes compared to WT littermate controls were plotted. All the values are the average of triple determinations with the SD indicated by error bars. *P<0.05 indicates a significant difference compared to mammary glands from WT littermate controls.