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Wnt5a is required for proper mammary gland development and TGF-β-mediated inhibition of ductal growth

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Transforming growth factor-β (TGF-β) plays an essential role in growth and patterning of the mammary gland, and alterations in its signaling have been shown to illicit biphasic effects on tumor progression and metastasis. We demonstrate in mice that TGF-β (Tgfβ) regulates the expression of a non-canonical signaling member of the wingless-related protein family, Wnt5a. Loss of Wnt5a expression has been associated with poor prognosis in breast cancer patients; however, data are lacking with regard to a functional role for Wnt5a in mammary gland development. We show that Wnt5a is capable of inhibiting ductal extension and lateral branching in the mammary gland. Furthermore, *Wnt5a*$^{-/-}$ mammary tissue exhibits an accelerated developmental capacity compared with wild-type tissue, marked by larger terminal end buds, rapid ductal elongation, increased lateral branching and increased proliferation. Additionally, dominant-negative interference of TGF-β signaling impacts not only the expression of Wnt5a, but also the phosphorylation of discoidin domain receptor 1 (Ddr1), a receptor for collagen and downstream target of Wnt5a implicated in cell adhesion/migration. Lastly, we show that Wnt5a is required for TGF-β-mediated inhibition of ductal extension in vivo and branching in culture. This study is the first to show a requirement for Wnt5a in normal mammary development and its functional connection to TGF-β.

KEY WORDS: Wnt5a, Mammary gland, TGF-β, Ddr1, Non-canonical Wnt, Mouse

INTRODUCTION

The complexity of mammary gland development presents a challenge in understanding the orchestration of signaling events that contribute to the formation of a finely patterned ductal network. Understanding how different signaling pathways are coordinated would provide better insight into how alterations in these events contribute to neoplastic transformation. The majority of mammary gland development is postnatal and involves the processes of patterning, branching morphogenesis, and differentiation. Pubertal and adult stages include ductal extension through the fat pad and the formation of secondary and higher order branches, while pregnancy and lactation result in the fully functional state of the mammary gland for the production and secretion of milk. Achieving an appropriately spaced ductal tree early in development is essential for milk-secreting lobuloalveolar units to form correctly during lactation. TGF-β, in particular, plays a key role in regulating growth and patterning of the mammary gland by acting as a negative regulator of ductal development and branching morphogenesis (Daniel et al., 1996; Ewan et al., 2002; Serra and Crowley, 2003; Serra and Crowley, 2005; Silberstein and Daniel, 1987; Wakefield et al., 2001).

Our laboratory previously showed that dominant-negative interference of TGF-β signaling (DNIIR) in the mammary gland results in accelerated ductal extension and increased lateral branching of the ductal tree compared with wild-type glands (Crowley et al., 2005; Joseph et al., 1999). Stemming from these studies, cDNA-based microarray and suppression-subtractive hybridization (SSH) assays were performed on glands from wild-type and DNIIR mice to identify genes with expression profiles that were altered when TGF-β signaling was interrupted. The hypothesis was that some of the genes that were regulated by TGF-β would mediate a subset of TGF-β inhibitory actions within the mammary gland. From this screen a gene involved in non-canonical Wnt signaling, *Wnt5a*, was identified. Wnt5a was of special interest because, like TGF-β, it has been suggested to act as a tumor suppressor in many tumor types, including those of the breast (Dejmek et al., 2005; Jonsson et al., 2002; Leris et al., 2005). Nevertheless, the role of Wnt5a in normal mammary development has gone largely unexplored.

The Wnt family of proteins consists of at least 19 members with functions that contribute to the regulation of a wide range of cellular processes, including proliferation and differentiation (Kikuchi et al., 2006; Veeman et al., 2003). Wnts have also been implicated in tumor formation (Brennan and Brown, 2004; Katoh, 2005). Wnts activate many signaling cascades, which can be divided broadly into two general categories: (1) the canonical, β-catenin-dependent pathway; and (2) the non-canonical β-catenin-independent pathway (Kikuchi et al., 2006; Kuhl et al., 2000; Veeman et al., 2003; Widelitz, 2005). In addition, it has been shown that some non-canonical Wnts can directly antagonize canonical signaling (Mikels and Nusse, 2006; Topol et al., 2003; Westfall et al., 2003). Wnt5a has been shown to signal through non-canonical signaling pathways in many cell types.

Previously, it was shown using northern blot analysis that *Wnt5a* is expressed at all stages of mammary development except lactation (Gavin and McMahon, 1992; Weber-Hall et al., 1994). During embryonic development, *Wnt5a* mRNA was localized by whole-mount in situ hybridization to a broad strip of mesenchyme underlying the area where the mammary placodes form (Chu et al., 2004). In adult virgin mice, expression was detected by northern blot in RNA isolated from cleared fat pads, suggesting that *Wnt5a* is expressed in the mammary stroma (Weber-Hall et al., 1994). Recently, it was shown using microarray screens that *Wnt5a* is preferentially expressed in the TEB relative to the mature ducts (Kouros-Mehr and Werb, 2006).

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Many of the observations that address the potential role of Wnt5a in the mammary gland come from in vitro studies using mammary cell lines. Early studies show that WNT5A expression decreases when cells of an immortalized human mammary epithelial cell line, HB2, are transferred from a two-dimensional monolayer culture to an embedded three-dimensional culture, and even more when three-dimensional cultures undergo branching morphogenesis in response to hepatocyte growth factor (HGF) treatment (Huguet et al., 1995). Overexpression studies show that WNT5A can abolish HGF-induced branching of HB2 cells in three-dimensional culture and enhance cell-to-collagen adhesion (Jonsson and Andersson, 2001). By contrast, siRNA-mediated repression of WNT5A enhances cell scattering, impairs cell-collagen interaction and increases motility of HB2 cells (Jonsson and Andersson, 2001). A correlation between discoidin domain receptor 1 (DDR1) phosphorylation and WNT5A expression has also been observed, and data exist that suggest a requirement of WNT5A for collagen-induced DDR1 activation/phosphorylation and proper mammary cell adhesion (Dejmek et al., 2003). Together, the results imply an inverse correlation between Wnt5a activity and branching.

In the current study, we show that TGF-β regulates the expression of Wnt5a, which in turn, mediates a subset of TGF-β inhibitory actions on ductal elongation and lateral branching during puberty and in non-pregnant adults. We demonstrate that Wnt5a exhibits inhibitory effects on mammary gland development similar to those of TGF-β. We go on to show the necessity of Wnt5a for normal ductal extension and branching, and subsequently provide evidence for a functional interaction between TGF-β and Wnt5a, through Ddr1 activation. We also show that Wnt5a is required for TGF-β-mediated inhibition of branching. The current study demonstrates a functional link between TGF-β and Wnt5a, which provides insight into the coordination of signaling events that contribute to mammary development and how alterations in these events might promote tumor progression.

MATERIALS AND METHODS

Mouse strains and maintenance

All mice in this study were maintained under the guidelines of the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. DNIIR mice and wild-type mice were previously described (Joseph et al., 1999). DNIIR and wild-type mice were administered 25 mM zinc sulfate in the drinking water for 2 weeks to induce DNIIR transgene expression. This time frame allowed for DNIIR expression before any phenotypic changes in branching occurred. Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine). *Wnt5a* disrupted mice (*Wnt5a*$^{tm1/Amc}$) were acquired from Jackson Laboratories and kept on a 129S7/C57B6 background to obtain *Wnt5a*$^{-/-}$ embryos. ICR/SCID mice were purchased from Taconic Farms.

Primary mammary cell isolation and culture

Adult virgin Balb/c mice (8-10 weeks of age) were the source of primary mammary epithelial cells and fibroblasts. Cell fractions were isolated by enzymatic dissociation and Percoll gradient density centrifugation as previously described (Xie and Haslam, 1997). Fibroblasts were plated in DMEM/F12 supplemented with 5% FBS, 100 μg/ml penicillin and 50 μg/ml streptomycin, were allowed to attach for 2 hours, were rinsed with 1×PBS, and were refreshed with growth medium. The epithelial cells were plated 1×10^5 cells/cm^2 in DMEM/F12 supplemented with 5% FBS, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, 100 μg/ml penicillin, 5 μg/ml streptomycin. Both epithelial cells and fibroblasts were grown to 80% confluence and serum-starved overnight before 5 ng/ml TGF-β1 treatment. For cycloheximide experiments, cells were pretreated overnight in basal conditions with 5 μg/ml cycloheximide. Serum-free medium was used for fibroblast cultures and basal medium (DMEM/F12 supplemented with 0.1 mM non-essential amino acids, 0.1 μg/ml insulin, 1 mg/ml fatty acid-free BSA (fraction V) and 100 μg/ml penicillin, 50 μg/ml streptomycin) was used for epithelial cultures. For IP studies, primary epithelial cells were plated on rat-tail collagen type I (BD Biosciences).

For three-dimensional cultures, primary mammary epithelial cells were suspended and plated in 24-well culture dishes in neutralized rat-tail collagen type I (2 mg/ml, 200 μl per well). Neutralized collagen was prepared according to the manufacturer's instructions (BD Biosciences). Cultures were allowed to gel for 30 minutes at 37°C, at which point 1 ml of overlay basal medium was added to each well. Treatments included 30 ng/ml HGF (R&D) alone or in combination with 40 ng/ml Wnt5a (R&D). For wild-type and *Wnt5a*$^{-/-}$ organoid experiments, cultures were treated with 30 ng/ml HGF alone or in combination with 5 ng/ml TGF-β1 (R&D). Analysis of branching was performed after 3 days.

RNA isolation and semi-quantitative RT-PCR

RNA isolation was performed using Trizol reagent. Relative levels of *Wnt5a* mRNA were determined using semi-quantitative RT-PCR. cDNA was synthesized from 2 μg total RNA using random primers. cDNA was then amplified using primers specific for *Wnt5a* and *18S*. Primer sequences are as follows: *Wnt5afwd* 5′-GAATCCCATTTGCAACCCCTCACC-3′, *Wnt5arev* 5′-GCTCCTCGTGTACATTTTCTGCCC-3′, *18Sfwd* 5′-ACGGAAGGGCACCACCAGG-3′, *18Srev* 5′-CACCAACTAAGAACGGCCATGC-3′. Amplification of cDNA was performed over varying cycles to arrive at product formation in the linear range.

Immunoprecipitation and western blot analyses

Protein from cells and tissue was extracted using RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Complete Mini) and sodium orthovanadate (Sigma). For Wnt5a analyses, 50 μg protein lysate was fractionated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with 0.2 μg/ml polyclonal Wnt5a antibody (R&D Systems, AF645) and rabbit anti-goat polyclonal secondary IgG-HRP (1:1000; Santa Cruz, sc-2922). To ensure equal protein loading between lanes, membranes were stripped and re-probed with anti-β-tubulin polyclonal antibody (1:1000; Santa Cruz H-325, sc-9104) and secondary goat anti-rabbit IgG-HRP (1:2000; Santa Cruz, sc-2054). Blots of the tissue lysate were also probed with α-HGF (Santa Cruz, sc-7949) to demonstrate the increase in HGF expression in the DNIIR phenotype. Also, blots of the cell culture experiments were probed with anti-Vimentin (Santa Cruz, sc-5565) to confirm fibroblast-rich populations. For immunoprecipitation studies, 500 μg protein lysate was pre-cleared with agarose-bound protein A (Vector, sp-0050) and 2 μg normal rabbit IgG (Santa Cruz, sc-2027), and immunoprecipitated with 1 μg polyclonal anti-Ddr1 antibody (Santa Cruz C-20, sc-532). The immunoprecipitated proteins were resolved on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with a rabbit polyclonal anti-phosphotyrosine antibody (Upstate, 06-427) to detect changes in the phosphorylation status of Ddr1. Membranes were stripped and re-probed with anti-Ddr1 as an internal control for loading.

Elvax slow-release pellet preparation and surgical implantation

The use of Elvax in slow-release delivery of proteins has been previously described (Silberstein and Daniel, 1987). Wnt5a pellets were prepared using 6 μg of Wnt5a protein and 20 μg BSA as a carrier. The final stock pellet was cut into ~1 mg pellets containing 170 ng Wnt5a protein. BSA control pellets were prepared with 20 μg BSA and no additional protein. Mice were anesthetized and incisions were made in the lower abdomen and along the hind legs to expose the #4 inguinal mammary glands. Pellets were implanted into the glands by making a pocket within the fat pad with fine surgical forceps and carefully placing the pellet within the pocket. BSA pellets were inserted into one gland, while Wnt5a pellets were implanted into the contralateral gland of the same mouse. Mammary glands were removed and evaluated 1 week after implantation of the pellets.

TGF-β1 pellets were prepared with 1 μg of protein. To test TGF-β1 pellet efficacy and demonstrate ductal inhibition by TGF-β1 in wild-type glands, TGF-β1 and BSA pellets were implanted into contralateral #4 glands of Balb/c mice at the onset of puberty just ahead of the leading edge of the ductal tree. Glands were evaluated after one week by whole-mount staining. For *Wnt5a*$^{-/-}$ experiments, surgical implantation of the pellets was

performed by clearing contralateral fat pads of endogenous epithelium followed by implantation of TGF-β1 or BSA pellets and transplanted *Wnt5a*$^{-/-}$ epithelium. The mammary glands were allowed to develop for 2 weeks and then removed and evaluated by whole-mount preparation.

BrdU, Ki67 and TUNEL analyses

Mice were injected with 60 μg/g body weight bromodeoxyuridine (BrdU) 2 hours before sacrifice. Mammary glands were removed, fixed in 4% paraformaldehyde overnight at 4°C and processed to paraffin block. Sections (5 μm) were cut, dried overnight and then stored at 4°C for BrdU and TUNEL analyses. For BrdU staining, sodium citrate (pH 6.0) antigen retrieval was performed, and sections were probed with a rat monoclonal anti-BrdU antibody (1:100; Abcam) and secondary biotinylated anti-rat IgG (1:500; Vector Laboratories, BA-4001). Detection was achieved using Cy-3 streptavidin (1:100; Zymed Laboratories, 43-4315) and either Yo-Pro (Invitrogen) or DAPI (Invitrogen, D-1306) nuclear counterstain. TUNEL staining was carried out using a fluorescent-based TdT FragEL DNA Fragmentation Kit (Calbiochem, QIA39). For proliferation studies on the mammary organoid experiments, three-dimensional embedded cultures were incubated with 10 mM BrdU for 1 hour before fixation with 4% paraformaldehyde and processing to section. For Ki67 immunofluorescence, sections were probed with a rabbit polyclonal anti-Ki67 antibody (10 μg/ml; NeoMarkers, Ab-4 RB-1510-P0) followed by a secondary biotinylated anti-rabbit IgG (1:500; Vector Laboratories, BA-1000). Detection was carried out with Cy-3 streptavidin followed by DAPI nuclear counterstaining.

Wnt5a$^{-/-}$ and wild-type anlagen isolation and transplantation

Wnt5a$^{-/-}$ and wild-type embryos [embryonic day 16.5 (E16.5)] were the source of the mammary tissue for renal capsule grafting and fat pad transplantation experiments. *Wnt5a*$^{-/-}$ embryos were phenotypically distinguishable from wild-type embryos. Briefly, mammary epithelial primordia were microdissected from female embryos as previously described (http://mammary.nih.gov). Mammary buds were then grafted onto mesenchyme and cultured overnight to allow the two tissue types to form one transplantable unit. Grafts were cultured on Whatman Nucleopore Track-Etch Membranes in DMEM/F12 medium supplemented with 10% FBS, 100 μg/ml penicillin and 50 μg/ml streptomycin. After overnight culture, grafts were either implanted under the kidney capsule of ICR/SCID mice (or syngeneic hosts) or within the cleared fat pad of ICR/SCID mice (DeOme et al., 1959). Wild-type and *Wnt5a*$^{-/-}$ mammary explants were surgically implanted within contralateral kidney capsules or fat pads of the same host to ensure the same hormonal environment for the transplanted tissue to develop. For the renal grafts, sometimes more than one graft was put into a single kidney. In some cases epithelium transplanted into the fat pad was allowed to fill the fat pad so that *Wnt5a*$^{-/-}$ cells could be isolated and used in cell culture experiments as described below.

RESULTS

TGF-β regulates the expression of Wnt5a mRNA and protein

The results from the cDNA-based microarray and SSH assays indicating that Wnt5a is regulated by TGF-β were validated by several approaches. The expression of Wnt5a was first examined in mammary glands of wild-type mice (those with intact TGF-β signaling) and compared to DNIIR mice (those that overexpress a dominant-negative form of the TGF-β type II receptor). The level of *Wnt5a* mRNA expression was reduced in DNIIR mammary glands relative to wild-type controls (Fig. 1A). In addition, western blot analysis of Wnt5a protein from wild-type and DNIIR mammary glands revealed considerably decreased protein levels in DNIIR

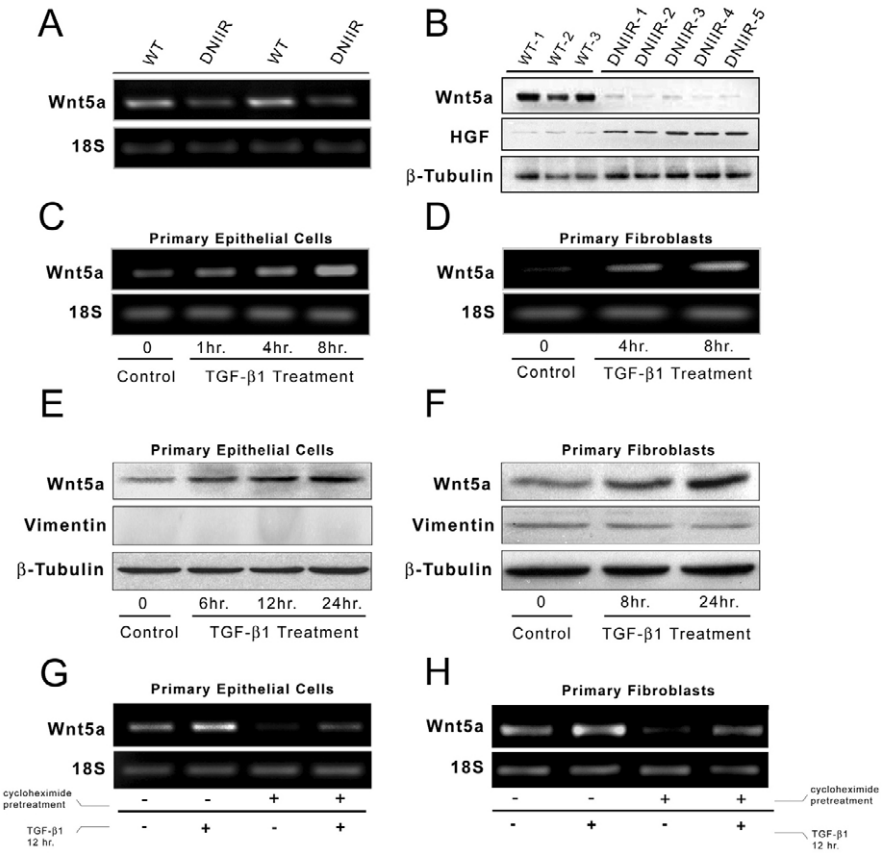

Fig. 1. TGF-β regulates Wnt5a expression in mice. (**A**) Semiquantitative RT-PCR of *Wnt5a* cDNA from wild-type and DNIIR mammary glands, illustrating reduced expression of *Wnt5a* mRNA in DNIIR glands relative to wild-type control glands. 18S was used to normalize for the amount of total RNA. PCR product is shown in the linear range (22 cycles for Wnt5a and 15 cycles for 18S). Wild-type and DNIIR labels represent mammary glands from individual mice administered $ZnSO_4$ for 2 weeks to induce DNIIR transgene expression. (**B**) Wnt5a and HGF protein levels in wild-type and DNIIR mammary glands depicted by western blot show reduced Wnt5a protein levels in DNIIR glands and elevated HGF levels. Wild-type and DNIIR labels represent mammary glands from separate mice. β-Tubulin was used to normalize for the amount of protein loaded. (**C-F**) Primary epithelial cells and fibroblasts isolated from 8-week-old adult virgin Balb/c mice were cultured and treated with TGF-β1. Treatment resulted in an induction of *Wnt5a* mRNA as measured by semiquantitative RT-PCR in both primary epithelial cells (C) and primary fibroblasts (D). Induction of Wnt5a was also seen at the protein level in both cell types (E,F). Vimentin expression was used as a marker for fibroblasts. (**G**,**H**) Mammary epithelial cells (G) and fibroblasts (H) were untreated (–) or pretreated (+) with cycloheximide followed by treatment with vehicle (–) or TGF-β1 for 12 hours (+), at which time RNA was extracted from the cells. The level of *Wnt5a* mRNA was determined by semi-quantitative RT-PCR. Treatment with TGF-β in the presence of cycloheximide resulted in an increase in *Wnt5a* mRNA, suggesting that TGF-β stimulates Wnt5a expression even in the absence of new protein synthesis.

mammary glands relative to wild-type glands (Fig. 1B). These results (Fig. 1A,B) demonstrate that Wnt5a mRNA and protein are diminished when TGF-β signaling is disrupted in the mammary gland. In light of the fact that lower Wnt5a expression could be a result of non-cell-autonomous or systemic effects of DNIIR in vivo, regulation of Wnt5a by TGF-β was next addressed by isolating primary epithelial cells and fibroblasts from the mammary glands of 8-week-old virgin Balb/c mice and subjecting them to TGF-β1 treatment over time in culture. TGF-β1 upregulated Wnt5a mRNA (Fig. 1C,D) and protein levels (Fig. 1E,F) in both epithelial cells and fibroblasts. Finally, to show that TGF-β regulates Wnt5a directly, in the absence of new protein synthesis, primary mammary epithelial cells and fibroblasts were left untreated or were pretreated with cycloheximide (Fig. 1G,H). Cells were then either untreated or treated with TGF-β1/ml and the levels of *Wnt5a* mRNA were measured by semi-quantitative RT-PCR. In the absence of cycloheximide, treatment with TGF-β resulted in an increase in the level of *Wnt5a* mRNA in both epithelial cells and fibroblast, as expected (Fig. 1G,H). In the presence of cycloheximide, the basal level of *Wnt5a* was reduced, suggesting that a labile factor is required for basal Wnt5a expression. Nevertheless, in the presence of TGF-β, *Wnt5a* mRNA levels were induced in both epithelial and fibroblast cells (Fig. 1G,H). The results suggest that TGF-β can directly activate transcription of *Wnt5a*.

Wnt5a inhibits ductal extension during puberty

As TGF-β regulated Wnt5a expression, we sought to investigate the role of Wnt5a in the mammary gland in vivo and determine whether Wnt5a had similar effects on branching as those reported for TGF-β. Elvax slow-release pellets containing BSA as a control or Wnt5a were implanted into contralateral #4 inguinal mammary glands of pubertal Balb/c mice at 3-4 weeks of age, in front of the terminal end buds of the developing ductal tree. After 1 week #4 inguinal glands receiving BSA pellets and their contralateral #4 glands receiving Wnt5a pellets were removed from mice to evaluate the effect of Wnt5a on ductal extension. In those glands that received Wnt5a pellets (Fig. 2B), ductal extension was inhibited compared with the contralateral gland that received BSA (Fig. 2A). Ductal extension was quantified by measuring the distance of the leading edge of the terminal end buds relative to the lymph node over the total length from the lymph node to the end of the fat pad. This ratio was expressed as a percentage of the terminal end bud distance traveled from node to the end of the gland. Quantification and statistical significance of the inhibition of ductal extension by Wnt5a is illustrated in Fig. 2C (n=11 mice; paired Student's t-test, P<0.05).

Terminal end buds are reduced in size in glands receiving Wnt5a

In addition to the inhibition of ductal extension during puberty, mammary glands that received Wnt5a pellets (Fig. 2E,G) exhibited a regression in terminal end bud size relative to contralateral glands that received BSA pellets (Fig. 2D,F). H&E stained sections showed that the TEBs were smaller in size and had lost normal structure (Fig. 2F,G). The effects elicited by Wnt5a on the end buds could be a result of decreasing proliferation, increasing apoptosis or a combination of both, so we carried out BrdU and TUNEL analysis on glands that received slow-release pellets and specifically analyzed proliferation and apoptosis in the terminal end buds between Wnt5a and BSA groups. BrdU incorporation in the terminal end buds demonstrated a fourfold decrease in proliferation in glands receiving Wnt5a pellets versus BSA pellets (Fig. 2H; n=31 end buds from BSA group, n=48 end buds from Wnt5a group, two-tailed Student's t-test, P<0.05). TUNEL staining did not indicate any significant changes in the percentage of cells undergoing apoptosis in the terminal end buds between Wnt5a and BSA groups (Fig. 2I; n=31 end buds from each group). The regression in size of the terminal end buds was therefore, at least in part, a result of a decrease in proliferation and not because of an increase in apoptosis.

Wnt5a inhibits branching morphogenesis in vivo and HGF-induced branching in vitro

The inhibitory effect of Wnt5a on ductal extension during puberty led us to speculate that Wnt5a might also inhibit lateral branching. To test this hypothesis, Wnt5a pellets were implanted within the ductal tree at the end of puberty, around 6 weeks of age. After 1 week, #4 inguinal glands that received Wnt5a pellets displayed fewer secondary and higher order branches in comparison to the contralateral glands that received BSA pellets (Fig. 3A-C). The number of lateral branches along the primary ducts was measured in three fields from each of the glands and the average number of secondary branches per 0.5 cm length of the primary branch was calculated. A paired Student's t-test indicated that the difference in branching was significant (Fig. 3C; n=4, P=0.002). To better quantify branching, we used a three-dimensional in vitro model consisting of primary mammary epithelial organoids embedded in a type I collagen matrix. Under basal conditions, organoids formed spherical 'cysts' without branches (Fig. 3D). Upon stimulation with HGF, sphere-shaped organoids formed branching processes after a few days (Fig. 3D). When treated with HGF in conjunction with Wnt5a, organoid branching was reduced (Fig. 3D). The extent of branching was quantified based on primary branch number and primary branch length. Primary branches were those extending directly from the sphere-shaped organoid. There was a statistically significant decrease in the number of primary branches (Fig. 3E; n=63 organoids per group, pooled from three independent experiments, P<0.05) and in the length of the branch (Fig. 3F; n=63 organoids per group, pooled from three independent experiments, P<0.05) when cultures were treated with Wnt5a. In addition, mammary organoids subjected to Wnt5a in combination with HGF displayed a decrease in proliferation as measured by BrdU incorporation compared with those organoids treated with HGF alone (Fig. 3G, n=32 organoids per group, pooled from two independent experiments, P<0.05). Together, these data illustrate the inhibitory effect of Wnt5a on branching. TGF-β has similar effects on primary and lateral branching, suggesting a model in which at least some of the effects of TGF-β on branching could be mediated by Wnt5a.

Wnt5a$^{-/-}$ mammary explants exhibit accelerated development

The experiments described until now establish the capacity of Wnt5a to inhibit ductal extension and lateral branching based on exogenous administration of Wnt5a, yet do not address a functional requirement for Wnt5a in development of the mammary gland. Phenotypic examination of the postnatal *Wnt5a*$^{-/-}$ mammary gland is not feasible as a result of severe developmental defects rendering the *Wnt5a*$^{-/-}$ mouse perinatal lethal. To address this issue, mammary buds and mesenchyme were harvested from E16.5 female embryos, cultured together overnight, and subsequently grafted under the kidney capsules of 3-week-old ICR/SCID mice, or transplanted into cleared fat pads of 3-week-old ICR/SCID mice. It was previously shown that embryonic development of *Wnt5a*$^{-/-}$ glands is normal (Chu et al., 2004), and we confirmed that result here (Fig. 4A). Subrenal grafting of the mammary tissue was an attractive strategy

to rescue *Wnt5a*$^{-/-}$ embryonic tissue, given that the kidney is a highly vascular organ and the take rate of the graft reaches nearly 100%. Also, evaluation of the absence of *Wnt5a* in both epithelial and stromal compartments is achieved using this strategy. By contrast, transplantation of tissue into cleared mammary fat pads addresses the absence of *Wnt5a* in the epithelium with intact expression in the stroma (DeOme et al., 1959). After 1 week under the kidney capsule, *Wnt5a*$^{-/-}$ explants exhibited accelerated development compared with wild-type explants that were inserted under the contralateral kidney of the same host (Fig. 4B; n=8). *Wnt5a*$^{-/-}$ mammary development was highlighted by the presence of larger terminal end buds, increased ductal extension and increased branching relative to wild-type explants. In addition, Brdu analysis of *Wnt5a*$^{-/-}$ and wild-type explants demonstrated a 2.2-fold increase in proliferation of the *Wnt5a*$^{-/-}$ explants compared with wild-type control explants (Fig. 4C; n=5 wild-type explants and n=6 *Wnt5a*$^{-/-}$ explants, Student's t-test, P=0.0005). Transplantation of wild-type and *Wnt5a*$^{-/-}$ mammary explants into cleared fat pads revealed that the absence of *Wnt5a* in the epithelium is enough to promote accelerated mammary development (Fig. 4D,E). Two weeks after surgical implantation of the explants into the fat pad, whole-mount evaluation of *Wnt5a*$^{-/-}$ transplants revealed a greater percentage of the fat pad filled,

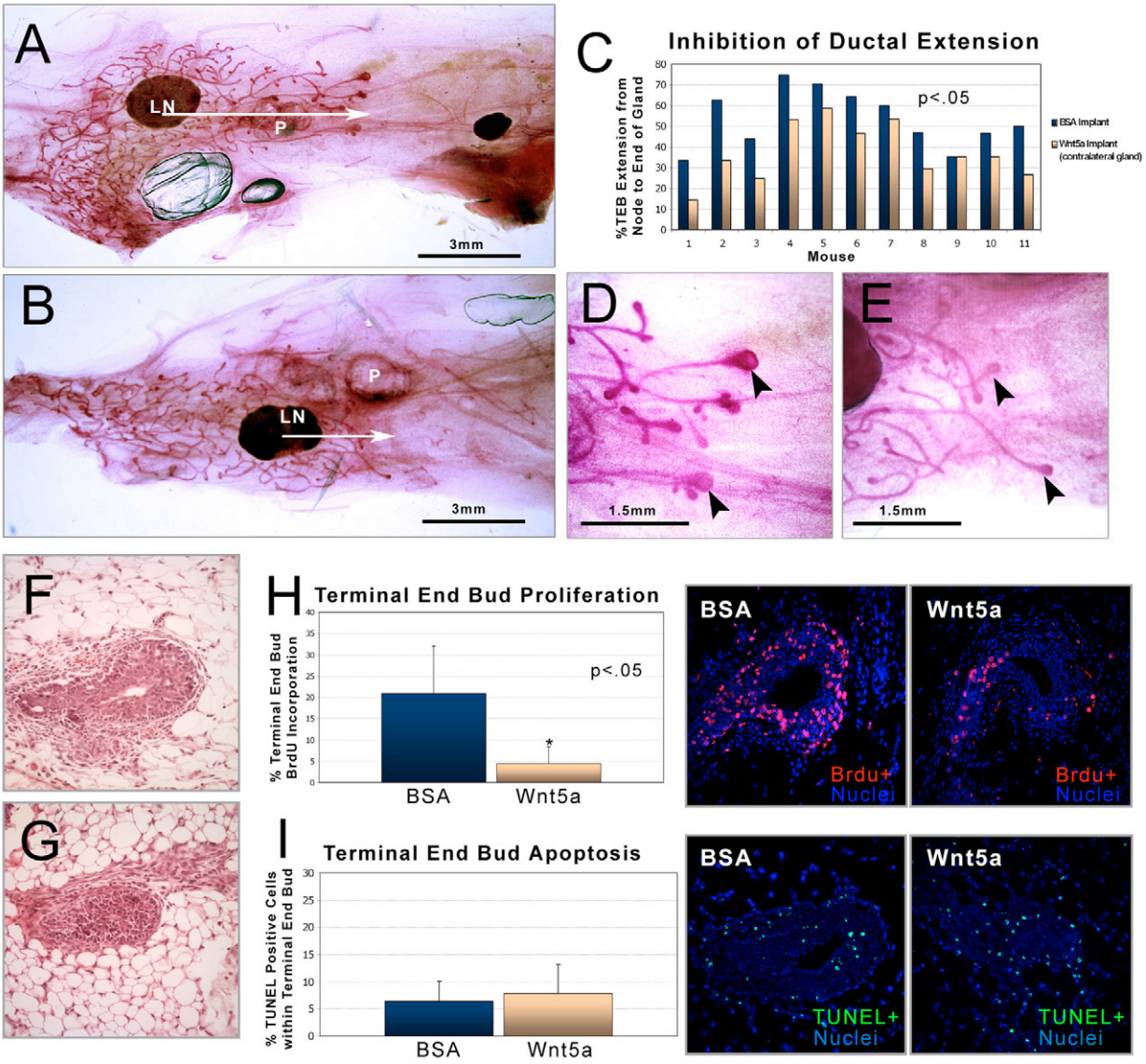

Fig. 2. Wnt5a inhibits ductal extension accompanied by diminished terminal end bud size and proliferation. (**A**) A representative whole-mount image of a #4 inguinal mammary gland with BSA pellet (P) depicts normal ductal extension through the fat pad. Horizontal arrow represents the distance from the lymph node to the end of the TEB. (**B**) A whole-mount image of the gland contralateral to the one shown in A illustrates the inhibition of ductal extension through the fat pad by Wnt5a pellet (P). (**C**) Quantification of ductal inhibition from 11 mice using a paired Student's t-test demonstrates that the inhibition is statistically significant (P<0.05). (**D**) Magnification of the terminal end bud size from control glands in A depicts the normal size of the end buds during puberty. (**E**) Magnification of the end buds in B demonstrates the smaller size of the end buds after Wnt5a exposure. (**F**,**G**) H&E staining of the terminal end buds from the BSA (F) and Wnt5a (G) groups illustrates the reduction in size of the terminal end buds with Wnt5a. (**H**) BrdU incorporation in the end bud. Exposure to Wnt5a resulted in a fourfold decrease in proliferation in the terminal end buds. Significance was determined by a Student's t-test (P<0.05). Representative images are shown to the right of the graph. (**I**) There was no statistically significant difference in apoptosis within the terminal end buds, determined by TUNEL staining.

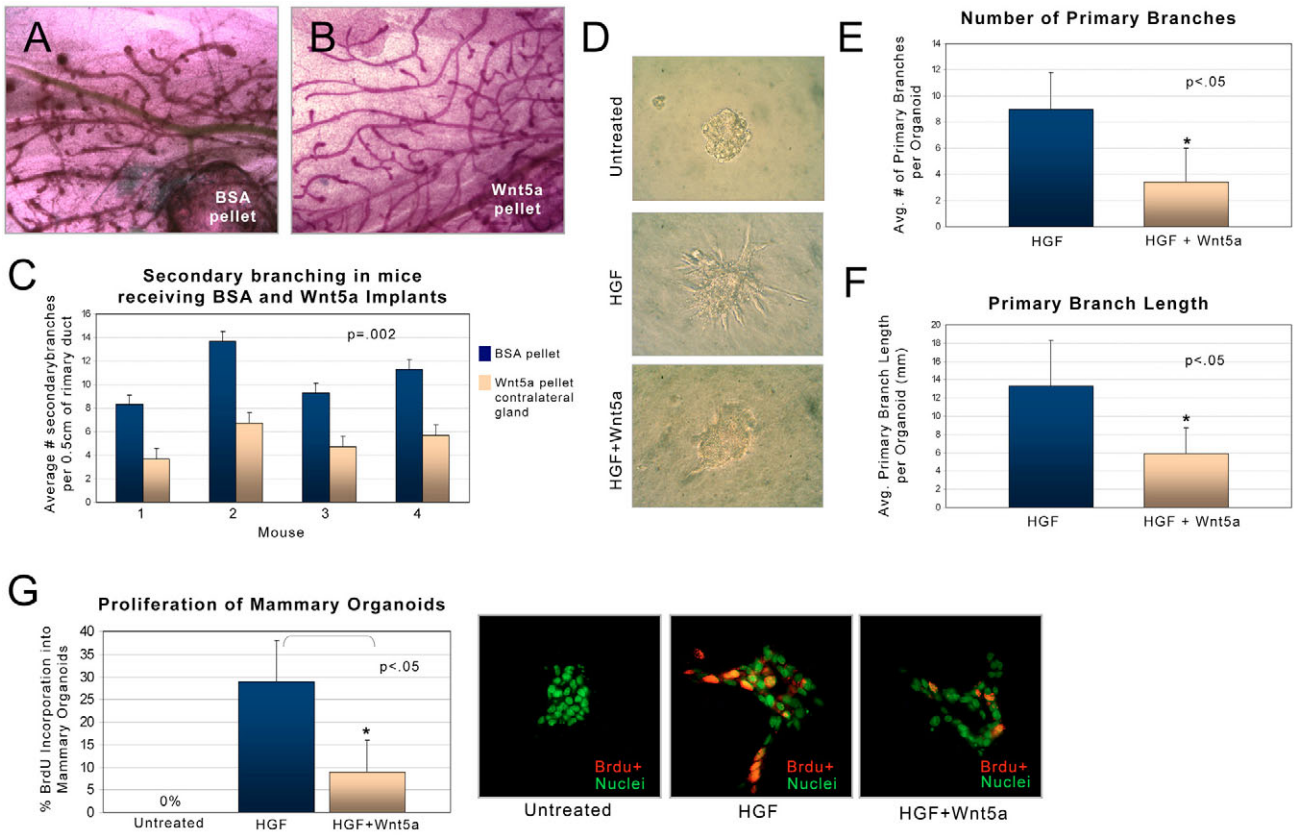

Fig. 3. Wnt5a inhibits lateral branching in vivo and HGF-induced branching morphogenesis in vitro. (**A-C**) Wnt5a inhibits lateral branching in vivo. Carmine staining of murine mammary glands treated with slow-release pellets containing BSA (A) or Wnt5a in the contralateral gland (B). The pellet is marked. (C) The average number of secondary branches per 0.5 cm of the primary branch was calculated from three separate fields for each gland. Error bars represent the standard deviation of the average from the three fields. A paired Student's *t*-test indicated that the differences were significant. (**D-G**) Wnt5a inhibits branching and growth in mammary organoids in culture. Phase contrast images showing primary mammary organoids untreated or treated with HGF alone or in combination with Wnt5a (D). Quantification of primary branch number demonstrates a statistically significant reduction in branching in cultures treated with Wnt5a compared to HGF alone, determined by a Student's *t*-test (E; $P<0.05$). Quantification of primary branch length shows that Wnt5a inhibited the extent of branching from the organoids, (F; $P<0.05$). Organoids treated with Wnt5a showed a statistically significant reduction in proliferation as determined by BrdU incorporation (G; $P<0.05$), illustrated to the right.

accompanied by an increase in terminal end bud size and an increase in lateral branching relative to the contralateral gland containing wild-type epithelium (Fig. 4D,E n=2 mice each containing wild-type and *Wnt5a*$^{-/-}$ epithelium in contralateral #4 glands), consistent with the results obtained from the subrenal grafting experiments. Together, these data suggest that *Wnt5a* from the epithelium facilitates proper growth and patterning of the mammary ductal tree by acting as a negative regulator of branching.

TGF-β activates discoidin domain receptor 1 via upregulation of Wnt5a

Ddr1 and Ddr2 are receptor tyrosine kinases that act as collagen receptors (Shrivastava et al., 1997; Vogel et al., 1997). Ddr1 is expressed at all stages of mammary development (Barker et al., 1995). Previously, it was shown that repression of WNT5A in HB2 cells results in enhanced cell motility and impaired binding to collagen (Jonsson and Andersson, 2001). Alterations in binding and migration after depletion of WNT5A were correlated with impaired phosphorylation of DDR1. Activation and subsequent phosphorylation of DDR1 was shown to require both collagen and WNT5A. Given that TGF-β regulates Wnt5a, we sought to investigate whether TGF-β could alter the activation status of Ddr1, and additionally, determine whether interruption of TGF-β signaling in the mammary gland affects Ddr1 phosphorylation in vivo. First, we determined if interruption of TGF-β signaling in the mammary gland impaired Ddr1 phosphorylation in vivo. Ddr1 was immunoprecipitated from protein lysates derived from DNIIR and wild-type mammary glands followed by western blot for phosphotyrosine or total Ddr1. We found that Ddr1 tyrosine phosphorylation was diminished in DNIIR mammary glands relative to wild-type glands, whereas total Ddr1 levels remained fairly constant (Fig. 5A). These results suggest that TGF-β signaling is required for proper Ddr1 activation. We next hypothesized that Wnt5a was required for TGF-β-mediated effects on Ddr1 activation. First, to show Ddr1 receptor activation in response to Wnt5a, primary epithelial cells cultured on type I collagen were treated with Wnt5a over a period of 6 hours. Immunoprecipitation of Ddr1 and western blotting for phosphotyrosine showed an increase in Ddr1 phosphorylation within 30 minutes of Wnt5a treatment (Fig. 5B). Primary epithelial cells (plated on type I collagen) treated with TGF-β1 also demonstrated an increase in Ddr1 phosphorylation, but after 30 hours. This activation corresponded to a time after the induction of Wnt5a protein levels seen at 24 hours (Fig. 5C). To test whether the activation of Ddr1 by TGF-β1 was directly mediated through the

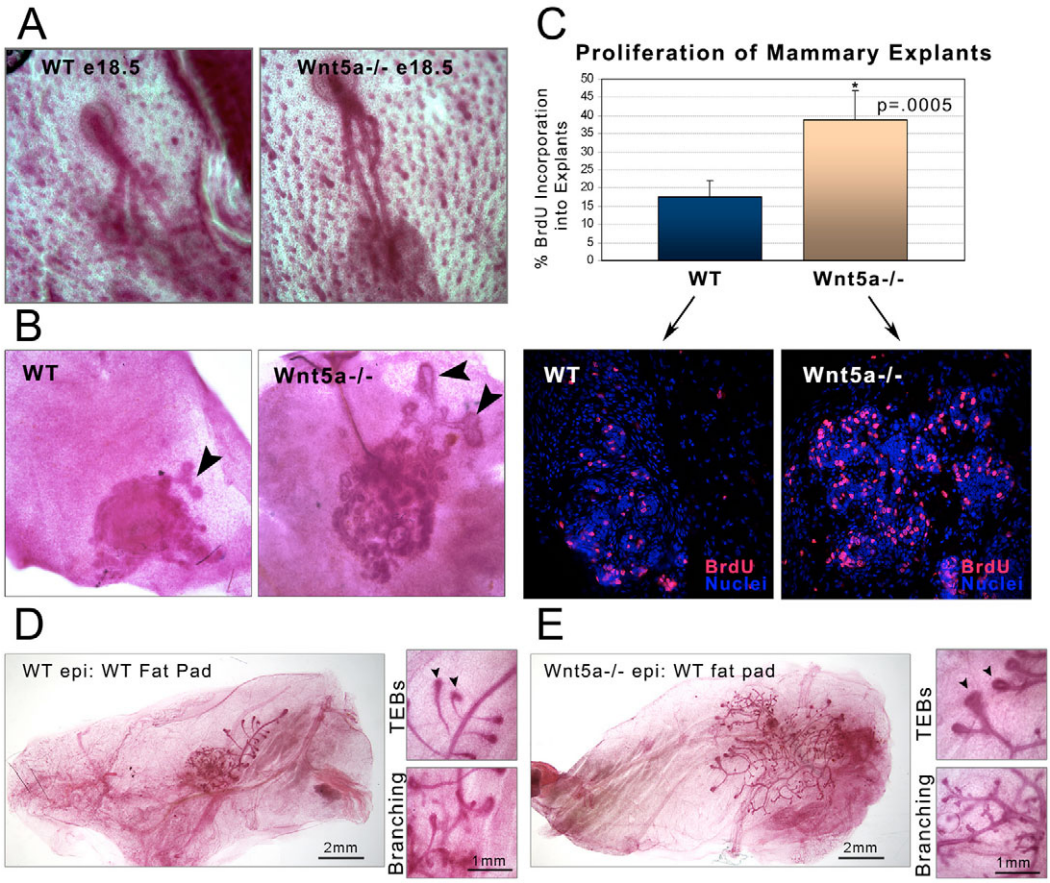

Fig. 4. Absence of Wnt5a accelerates mammary development in mice. (**A**) Carmine-stained whole-mount #1 gland from E18.5 wild-type and *Wnt5a*$^{-/-}$ mice. (**B**) Carmine-stained mammary tissue that was grafted under the kidney capsule of 3-week-old ICR/SCID mice demonstrates accelerated development in *Wnt5a*$^{-/-}$ tissue compared with wild-type tissue after 1 week. Increased ductal invasion, extensive branching and larger terminal end buds were noted in the *Wnt5a*$^{-/-}$ tissue (arrowheads). (**C**) Brdu analysis of mammary tissue transplanted under the kidney capsule demonstrates a twofold increase in proliferation of *Wnt5a*$^{-/-}$ mammary tissue compared with wild type (P=0.0005). (**D**,**E**) Whole-mount staining of mammary tissue inserted into cleared fat pads of 3-week-old ICR/SCID hosts shows accelerated mammary development of Wnt5a$^{-/-}$ epithelium (E) compared with wild-type epithelium (D) after 2 weeks of development. Arrowheads indicate terminal end buds (TEBs).

upregulation of Wnt5a, *Wnt5a*$^{-/-}$ epithelium was transplanted into cleared fat pads of wild-type mice and allowed to fill the fat pad. The *Wnt5a*$^{-/-}$ cells were then isolated and cultured on type I collagen followed by treatment with TGF-β1 for up to 48 hours. Compared with wild-type primary epithelium, which exhibited an increase in Ddr1 phosphorylation after TGF-β1 treatment, *Wnt5a*$^{-/-}$ cells failed to undergo Ddr1 phosphorylation in response to TGF-β1 (Fig. 5C). These findings illustrate that TGF-β can affect the phosphorylation/activation status of Ddr1 through the regulation of Wnt5a.

TGF-β1 is unable to inhibit ductal extension and branching in the absence of Wnt5a

Data up until this point offer evidence that Wnt5a exhibits a striking similarity to TGF-β by its ability to inhibit various aspects of mammary development, that Wnt5a is required for proper mammary gland development, and that TGF-β and Wnt5a may have a functional connection through Ddr1. Based on this, we decided to investigate whether TGF-β requires Wnt5a to inhibit mammary gland development. Elvax slow-release pellets containing TGF-β1 and BSA were implanted into contralateral cleared fat pads of pubertal ICR/SCID mice, which also received *Wnt5a*$^{-/-}$ epithelium. First, to ensure that the TGF-β1 pellets were active, BSA and TGF-β1 pellets were inserted into wild-type mice at 3 weeks of age in front of the developing end buds. TGF-β1 pellets inhibited ductal extension and terminal end bud size compared with the contralateral glands that received BSA pellets (Fig. 6A, n=4 wild-type mice each with BSA and TGF-β pellets in contralateral glands). Furthermore, proliferation as measured by Ki67 staining was dramatically reduced in TGF-β-treated end buds (Fig. 6C), confirming the activity of the pellets. Next, *Wnt5a*$^{-/-}$ tissue was examined. In glands containing *Wnt5a*$^{-/-}$ epithelium, TGF-β1 was unable to inhibit ductal extension, and the extent of branching was similar to the contralateral glands that received BSA pellets (Fig. 6B, n=4 mice reconstituted with *Wnt5a*$^{-/-}$ epithelium each with BSA and TGF-β pellets in contralateral glands). Proliferation in the end buds as measured by Ki67 staining was not different in BSA or TGF-β-treated end buds (Fig. 6D). The results suggest TGF-β activity on the end bud requires Wnt5a. Next, primary wild-type and *Wnt5a*$^{-/-}$ organoids were generated and embedded in a three-dimensional type I collagen matrix, then treated with HGF alone or in combination with TGF-β1 (Fig. 6E,F). TGF-β1 was unable to inhibit HGF-induced branching morphogenesis in *Wnt5a*$^{-/-}$ organoids (Fig. 6F) compared with the

clear inhibition of branching in wild-type organoids (Fig. 6E). Quantification of the number of primary branches revealed a sixfold reduction in the number of branches in TGF-β-treated wild-type organoids compared with those treated with HGF alone. Statistical differences in branch number were not detected in HGF-treated versus HGF+TGF-β1-treated *Wnt5a*$^{-/-}$ organoids (Fig. 6G, *n*=30 organoids per group, pooled from two independent experiments). In addition, *Wnt5a*$^{-/-}$ organoids branched under basal conditions in the absence of HGF, which was not seen in wild-type cultures (Fig. 6D, upper row). Branching of *Wnt5a*$^{-/-}$ organoids, both in the presence and absence of HGF, was accompanied by cell scattering into the surrounding matrix. Together, these data strongly suggest that Wnt5a is required to mediate many of the inhibitory effects of TGF-β on branching in the mammary gland.

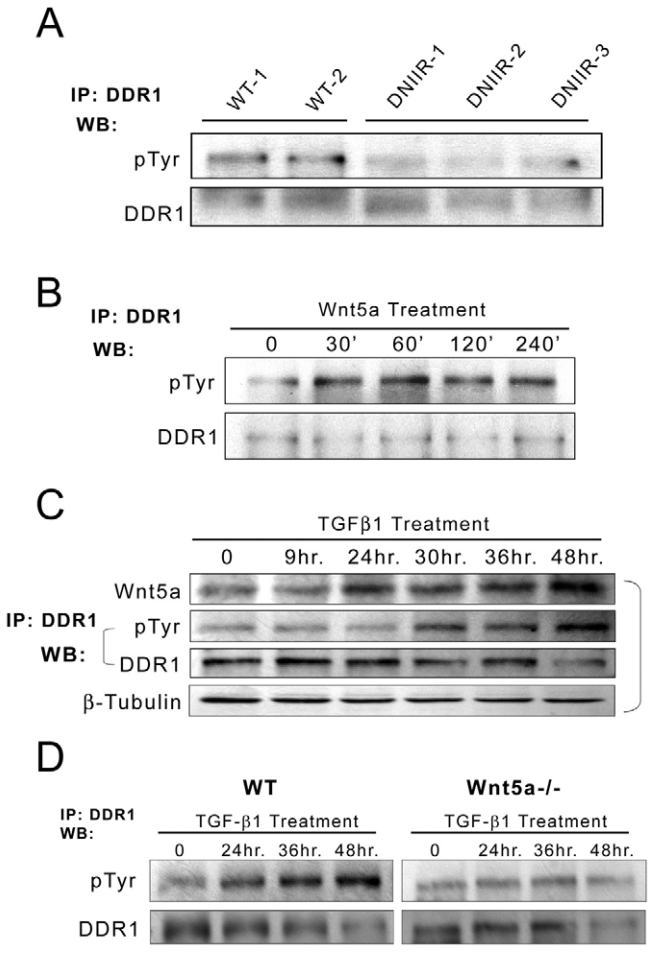

Fig. 5. TGF-β activates discoidin domain receptor 1 via upregulation of Wnt5a in mice. (**A**) Immunoprecipitation of Ddr1 from protein lysate of DNIIR and wild-type mammary glands and western blotting for phosphotyrosine demonstrates diminished Ddr1 tyrosine phosphorylation in DNIIR glands compared with wild-type glands. (**B**) Primary epithelial cells cultured on type I collagen were treated with Wnt5a. Immunoprecipitation of Ddr1 and western blotting for phosphotyrosine shows an increase in Ddr1 phosphorylation within 30 minutes of Wnt5a treatment. (**C**) Western blots show TGF-β1 is able to induce Ddr1 phosphorylation in primary epithelial cells after 30 hours, following induction of Wnt5a protein levels seen at 24 hours. (**D**) IP western blotting illustrates *Wnt5a*$^{-/-}$ epithelial cells cultured on type I collagen were unable to undergo Ddr1 phosphorylation in response to TGF-β1 treatment.

DISCUSSION

In this study, we show for the first time that TGF-β regulates the expression of Wnt5a in the mammary gland, and furthermore, that Wnt5a plays a role in the maintenance of proper ductal development. By exogenous administration of Wnt5a, we demonstrate that Wnt5a inhibits mammary gland development by suppressing ductal extension, terminal end bud proliferation and lateral branching, much like TGF-β (Pierce, et al., 1993; Silberstein and Daniel, 1987). Moreover, loss-of-function experiments involving subrenal grafting and fat pad transplantation of *Wnt5a*$^{-/-}$ mammary tissue revealed a requirement for Wnt5a in normal mammary development. In the absence of Wnt5a, development was accelerated, marked by the presence of larger terminal end buds, more extensive ductal invasion, increased lateral branching and elevated proliferation within the epithelium relative to wild-type glands. Additionally, we were able to identify a functional connection between TGF-β and Wnt5a. TGF-β facilitated the phosphorylation of Ddr1 via the regulation of Wnt5a expression and interruption of TGF-β signaling in vivo reduced both Wnt5a expression and the phosphorylation status of Ddr1. Lastly, we show that TGF-β1 is unable to inhibit ductal extension and growth in *Wnt5a*$^{-/-}$ mammary transplants or branching in *Wnt5a*$^{-/-}$ organoids, suggesting that Wnt5a is required for TGF-β-mediated inhibition of mammary development.

A key finding made here is that Wnt5a is required for many of the effects of TGF-β on branching. TGF-β pellets placed in front of the end bud normally inhibit ductal extension through the fat pad accompanied by a decrease in end bud size; however, *Wnt5a*$^{-/-}$ epithelium was resistant to the inhibitory effects of TGF-β. Additionally, HGF-induced branching in *Wnt5a*$^{-/-}$ organoids was not inhibited by TGF-β. Wild-type organoids, by contrast, exhibit a dramatic reduction in HGF-induced branching when treated with TGF-β. This is the first evidence that TGF-β requires a secondary effector, Wnt5a, for some of its inhibitory actions in the mammary gland.

It is possible, based on our data and those of other studies (Dejmek et al., 2003; Jonsson and Andersson, 2001), that the inhibitory effects of TGF-β and Wnt5a are based on the ability of these proteins to modify cell adhesive properties, thus dictating the proliferative or migratory capacity of the epithelium in response to other signals. Ddr1, which we demonstrate can be activated by TGF-β via upregulation of Wnt5a, has been shown to inhibit migration of breast cancer cells in vitro (Hansen et al., 2006) and tubulogenesis/migration of MDCK cell in three-dimensional culture (Wang et al., 2005; Wang et al., 2006). Moreover, data suggest that Ddr1 tyrosine kinase activity can mediate the expression of MMPs (Hou et al., 2002), which are involved in the orchestration of mammary development. It is known that one of the main functions of TGF-β is to mediate cell adhesion and shape (Massague et al., 1992; Sporn and Roberts, 1988). It has also been suggested that WNT5A is a key regulator of mammary cell adhesion through its ability to act as a co-factor for the activation and phosphorylation of DDR1 (Dejmek et al., 2003; Jonsson and Andersson, 2001). At 3 months of age, *Ddr1*-null mammary glands exhibit an increase in lateral branching and proliferation when compared with wild-type glands with some similarities to the effects of dominant-negative interference of TGF-β signaling or loss of Wnt5a, suggesting that TGF-β, Wnt5a and Ddr1 may interact to regulate proliferation and branching in the adult mammary gland (Crowley et al., 2005; Joseph et al., 1999; Vogel et al., 2001). We previously showed that dominant-negative interference of TGF-β signaling results in elevated expression of HGF (Joseph et al., 1999). Overexpression of

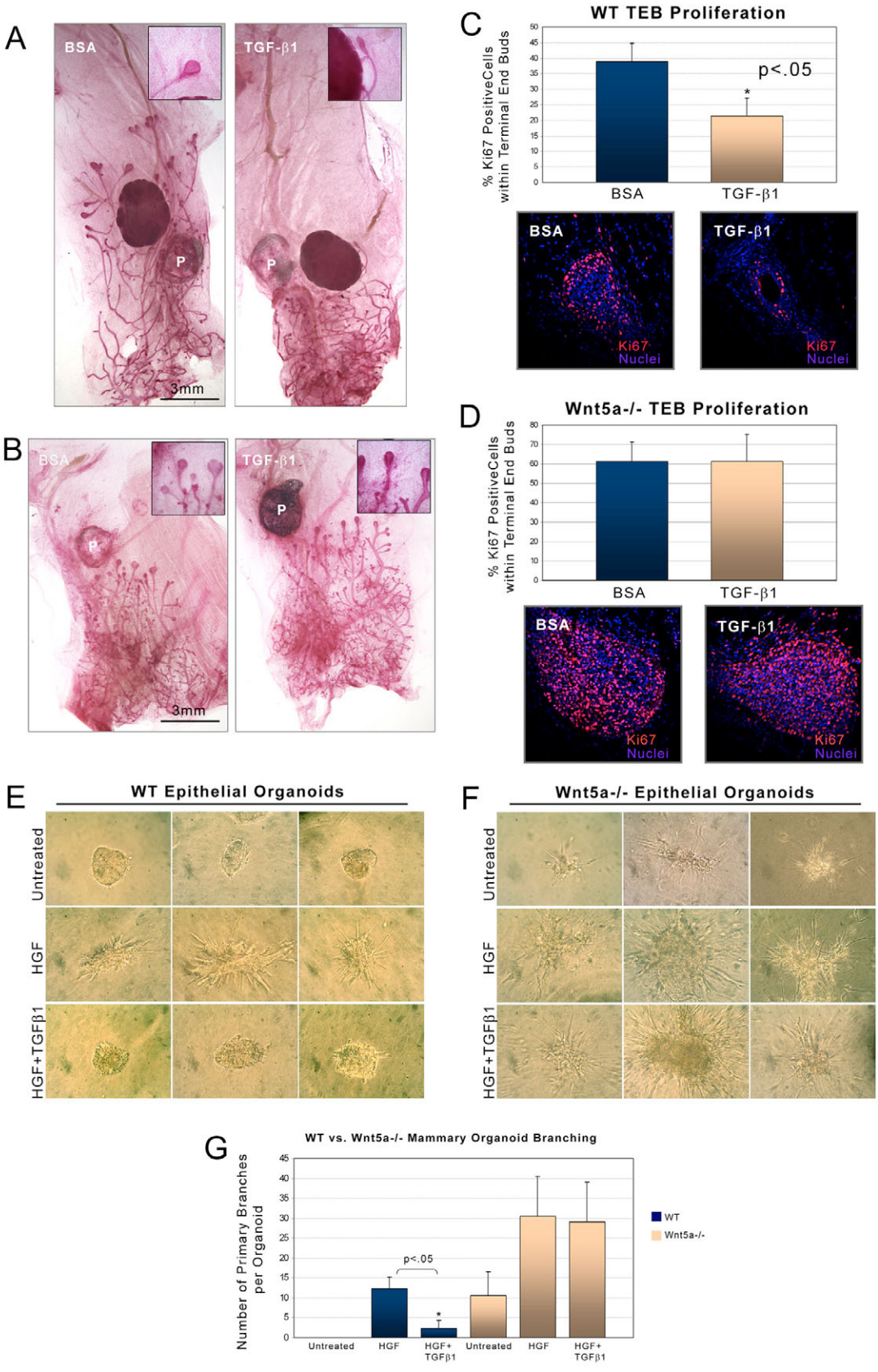

Fig. 6. In mice, TGF-β1 is unable to inhibit ductal growth of *Wnt5a*$^{-/-}$ epithelium in vivo and branching of *Wnt5a*$^{-/-}$ organoids in vitro. (**A**) Carmine-stained BSA-treated and the contralateral TGF-β-treated wild-type mammary gland confirms that the TGF-β1 pellets (P) are functional and result in degeneration of the end bud and inhibition of ductal elongation. Insets represent magnified images of the end buds. (**B**) Carmine-stained glands reconstituted with *Wnt5a*$^{-/-}$ epithelium. The left gland was treated with BSA and the right contralateral gland was treated with TGF-β1. TGF-β is unable to inhibit the end bud in the absence of Wnt5a. Insets represent magnified images of the end buds. (**C**) Ki67 staining in the end buds of wild-type mice either treated with BSA or TGF-β. Treatment with TGF-β resulted in reduced staining relative to BSA-treated glands. (**D**) Ki67 staining in the end buds of *Wnt5a*$^{-/-}$ reconstituted glands treated with BSA or TGF-β. Staining was similar in BSA- and TGF-β-treated end buds. (**E**) Phase contrast images of wild-type mammary organoid preparations untreated or treated with HGF or HGF and TGF-β1 shows TGF-β-mediated inhibition of HGF-induced branching. Three representative images are shown for each condition. (**F**) *Wnt5a*$^{-/-}$ organoids were untreated or treated with HGF or TGF-β and HGF. Untreated *Wnt5a*$^{-/-}$ cultures demonstrated increased branching relative to wild-type cultures. TGF-β failed to inhibit branching in the absence of Wnt5a. Three representative images are shown for each condition. (**G**) Primary branching was quantified in wild-type and *Wnt5a*$^{-/-}$ cultures untreated or treated with HGF or TGF-β and HGF. In wild-type cultures treatment with TGF-β resulted in a statistically significant decrease in branching. TGF-β did not inhibit branching in *Wnt5a*$^{-/-}$ cultures.

DDR1 (Wang et al., 2005) or WNT5A (Jonsson and Andersson, 2001) inhibits HGF-induced branching in vitro. It seems likely that elevated HGF expression from the stroma in conjunction with a decrease in Wnt5a expression and subsequent loss in Ddr1 activation might facilitate branch formation and result in increased branching seen when TGF-β signaling is interrupted. These observations create a potential mechanism for the regulation of branching by TGF-β through its regulation of both negative and positive mediators of branching, Wnt5a and HGF.

Wnts activate many signaling cascades that can be broadly divided into two general categories: (1) the canonical, β-catenin pathway; and (2) the non-canonical pathway (Kikuchi et al., 2006; Kuhl et al., 2000; Veeman et al., 2003; Widelitz, 2005). Wnt5a has been shown to act primarily through non-canonical signaling pathways. Among the non-canonical pathways, Wnt5a has been shown to activate both the Wnt/Ca^{+2} and planar cell polarity (PCP) pathways. Wnt5a can also antagonize canonical signaling but the mechanism is not clear and varies between cell types. The non-canonical signaling pathways are not very well characterized in general and not at all understood in the context of normal mammary development. However, the role of canonical Wnt signaling has been studied in mouse mammary gland development (Brennan and Brown, 2004). Expression of a Wnt reporter, TOPGAL, and characterization of mice with mutations or deletions of components of the canonical pathway suggest that canonical signaling is important in early embryonic development of the mammary gland and then again during pregnancy (Chu et al., 2004; Hsu et al., 2001; Brisken et al., 2000; Tepera et al., 2003). Although several canonical signaling Wnts are expressed during puberty and in the adult mammary gland, there is no evidence that canonical signaling is required for normal development at these stages; nevertheless, inappropriate canonical signaling in adult mice results in hyperplasia or metaplasia in the ducts and is correlated with tumorigenic transformation (Benhaj et al., 2006; Gavin and McMahon, 1992; Imbert et al., 2001; Kouros-Mehr and Werb, 2006; Miyoshi et al., 2002). Wnt5a has been shown to antagonize canonical signaling in several cell types, and it is possible that its role in normal development is to suppress inappropriate canonical signaling. Furthermore, even though several non-canonical signaling Wnts are expressed in the mammary gland (Benhaj et al., 2006; Kouros-Mehr and Werb, 2006), the fact that loss of Wnt5a alone is sufficient to alter normal branching and growth suggests that Wnt5a has unique roles in mammary development. It should also be noted that experiments in which *Wnt5a*$^{-/-}$ epithelium is transplanted into the cleared fat pads of wild-type mice indicate that Wnt5a synthesized in the epithelium is required for normal branching and growth. Although Wnt5a expression has been detected in the stroma, stromal Wnt5a alone is not sufficient to mediate normal growth and branching.

This study is the first to show a functional requirement for Wnt5a in the mammary gland and the first to show that Wnt5a acts to mediate many of the effects of TGF-β on branching. We demonstrate that Wnt5a is required for TGF-β to regulate mammary branching and phosphorylation of Ddr1. The task at hand lies in identifying downstream signaling targets of Wnt5a and Ddr1 that could provide insight into how these factors regulate development of the mammary gland. Future work will be directed at understanding the role of Wnt5a in tumor formation and progression.

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