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Progesterone and Wnt4 control mammary stem cells via myoepithelial crosstalk

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Abstract

Ovarian hormones increase breast cancer risk by poorly understood mechanisms. We assess the role of progesterone on global stem cell function by serially transplanting mouse mammary epithelia. Progesterone receptor (PR) deletion severely reduces the regeneration capacity of the mammary epithelium. The PR target, receptor activator of Nf-κB ligand (RANKL), is not required for this function, and the deletion of Wnt4 reduces the mammary regeneration capacity even more than PR ablation. A fluorescent reporter reveals so far undetected perinatal Wnt4 expression that is independent of hormone signaling. Pubertal and adult Wnt4 expression is specific to PR$^+$ luminal cells and requires intact PR signaling. Conditional deletion of Wnt4 reveals that this early, previously unappreciated, Wnt4 expression is functionally important. We provide genetic evidence that canonical Wnt signaling in the myoepithelium required PR and Wnt4, whereas the canonical Wnt signaling activities observed in the embryonic mammary bud and in the stroma around terminal end buds are independent of Wnt4. Thus, progesterone and Wnt4 control stem cell function through a luminal–myoepithelial crosstalk with Wnt4 acting independent of PR perinatally.

Keywords canonical Wnt signaling; hormones; mammary stem cells; myoepithelium; paracrine

Subject Categories Development & Differentiation; Signal Transduction; Stem Cells

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Introduction

The two key ovarian hormones, 17-β-estradiol (E2) and progesterone, regulate postnatal mammary gland development but also promote carcinogenesis in this organ. They act via nuclear receptors, the estrogen receptor α (ER) and the progesterone receptor (PR), respectively, which are expressed in between 30 and 50% of the mammary epithelial cells in the inner, luminal layer (Clarke *et al*, 1997). It was proposed that they impinge on ER-/PR-negative mammary stem cells by paracrine mechanisms (Tanos & Brisken, 2008). Experimental evidence for this model was provided with fluorescence-activated cell sorting (FACS)-based approaches (Asselin-Labat *et al*, 2010; Joshi *et al*, 2010). The single-cell-based methods have been used to characterize mammary epithelial cell populations and to establish a cellular hierarchy within the mammary epithelium. Among dissociated CD24$^+$ mouse mammary epithelial cells, the cell populations with high surface expression of integrin β1 (CD29^{hi}) or integrin α6 (CD49f^{hi}) were enriched for cells with the ability to establish new milk ducts in mammary fat pads surgically cleared of their endogenous epithelium and were hence considered bipotent mammary stem cells able to give rise to both luminal and basal/myoepithelial cell lineage (Shackleton *et al*, 2006; Stingl *et al*, 2006). These cells express basal/myoepithelial markers such as cytokeratin 5 and 14, smooth muscle actin, and laminin (Shackleton *et al*, 2006; Stingl *et al*, 2006) and their numbers increase during pregnancy and after stimulation with E2 and progesterone (Asselin-Labat *et al*, 2010; Joshi *et al*, 2010) and decrease upon ovariectomy or anti-estrogen treatment (Asselin-Labat *et al*, 2010).

However, the physiological relevance of the dramatic expansion of these bipotent stem cells in response to hormones (Asselin-Labat *et al*, 2010; Joshi *et al*, 2010) is questioned by lineage-tracing experiments showing that postnatal mammary gland development is largely driven by luminal and basal/myoepithelial lineage-restricted stem cells (Taddei *et al*, 2008; Zeng & Nusse, 2010; Van Keymeulen *et al*, 2011; Rios *et al*, 2014). The lineage-restricted stem cells are not amenable to study by the single stem cell assays where the normal stem cell niche and its microenvironment are disrupted.

Recurrent peaks in serum progesterone levels are linked to menstrual cycles which are an important risk factor for breast carcinogenesis (Brisken, 2013). Moreover, most cell proliferation occurs in the luminal compartment and breast cancers arise from luminal and/or luminal progenitor cells (Molyneux *et al*, 2010). This begs the question how important progesterone is to mammary stem cell

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function in the intact tissue context and through which signaling pathways it impinges on different types of mammary stem cells, that is, bipotential, luminal restricted, and basal restricted.

Mammary epithelium was shown to serially engraft cleared mammary fat pads for up to seven cycles (Daniel, 1973). This assay, which is based on the engraftment of an intact piece of mammary epithelium, preserves the epithelial architecture with its associated extracellular matrix, fibroblasts, and immune cells. It is currently the only way to assay comprehensively the mammary regeneration potential reflective of multiple types of stem and progenitor cells. We combine this assay with different genetic mutant strain to define the relative contributions of progesterone signaling and its downstream mediators RANKL and Wnt4 to the regenerative potential of the mammary epithelium.

Results

PR signaling for mammary epithelial self-renewal

To assess the role of PR signaling in mammary stem cell control, we serially engrafted pieces of intact mammary epithelium from the $PR^{-/-}$ and wild-type (WT) littermates into contralateral mammary fat pads surgically cleared of their endogenous epithelium (Fig 1A). This approach rather than injection of limiting dilutions of dissociated cell populations was chosen so that the physiological interactions between the stem/progenitor cells and their microenvironment in the mammary epithelium niche would be preserved and the function of all types of stem cells, the bipotential, the luminal-restricted, and the basal-restricted stem cells, could be evaluated. To unequivocally distinguish the engrafted cells from the endogenous epithelium that may inadvertently be left after surgery, we used donors that ubiquitously express the enhanced green fluorescent protein (EGFP) (Okabe *et al*, 1997). To ensure that comparable amounts of mutant (*MT*) and *WT* donor tissue with comparable amounts of epithelium were engrafted, we dissected pinhead-sized fragments from the inguinal glands near the lymph node on the side proximal to the teat. Eight to 12 weeks after grafting, recipients were sacrificed and the extent of outgrowth in the engrafted mammary glands was determined. Pieces of mammary tissue resulting from the contralateral $PR^{-/-}$ and *WT* grafts were dissected and retransplanted (Fig 1A). The *WT* epithelium completely reconstituted most of the fat pads over 4 serial transplant cycles, as expected, but the $PR^{-/-}$ epithelium ceased to reconstitute the mammary gland by the 3rd cycle (Fig 1B, C, and H).

Paracrine mediators of PR signaling in mammary epithelial self-renewal

The TNF-α family member, RANKL, was previously implicated in the paracrine control of mammary stem cells by hormones on the basis of use of dissociated individual stem cell assays (Asselin-Labat *et al*, 2010). To determine the functional importance of RANKL in epithelial self-renewal, we serially engrafted intact mammary epithelium derived from the $RANKL^{-/-}$ and the $RANKL^{+/+}$ mice into cleared contralateral mammary fat pads. *WT* epithelium fully reconstituted fat pads in most hosts over 4 serial transplants and grew as expected (Fig 1D and E). Unexpectedly, $RANKL^{-/-}$ epithelia had the same regeneration capacity (Fig 1D and E). The only significant difference was that the *MT* grafts generated fewer side branches (Fig 1D and insets), consistent with the reported proproliferative activity of RANKL and its role in side branching (Beleut *et al*, 2010).

We tested the role of Wnt4 since evidence has accumulated that Wnt signaling is important for mammary stem cell function (Cai *et al*, 2014; Kessenbrock *et al*, 2013; Liu *et al*, 2004; van Amerongen *et al*, 2012; Wang *et al*, 2014; Zeng & Nusse, 2010) and Wnt4 is a key paracrine mediator of progesterone action (Brisken *et al*, 2000). As $Wnt4^{-/-}$ mice die at birth (Vainio *et al*, 1999), intact mammary epithelial buds from E12.5/E13.5 embryos were used for the initial engraftment. It has been reported that embryonic epithelia have more stem cells than postnatal epithelia (Spike *et al*, 2012). Notwithstanding, we noticed that epithelial tissue isolated from the $Wnt4^{+/+}$ and the $Wnt4^{+/-}$ embryos reconstituted completely the mammary gland to the same extent as the *WT* epithelia from postnatal mammary glands through three transplantation cycles (Fig 1C, E, and G). However, the $Wnt4^{-/-}$ epithelium only established 50% of the fat pad in the first cycle and was reduced to 10% by the third cycle (Fig 1F and G). The much more significant impairment of reconstitution capacity of the $Wnt4^{-/-}$ versus the $PR^{-/-}$ grafts compared to their respective contralateral controls (Fig 1H) points to a key role for Wnt4 in the maintenance of the mammary stem cell function and indicates that PR is not exclusively controlling Wnt4 expression. RANKL

Figure 1. PR, RANKL, and Wnt4 and their role in the regenerative capacity of the mammary gland.

A Experimental scheme. Mammary tissue fragments dissected from wild-type (WT) or mutant (MT) donor mice were engrafted to contralateral mammary fat pads of $RAG1^{-/-}$ recipient mice surgically divested of the endogenous epithelium. Between 8 and 12 weeks later, the engrafted glands were assessed by fluorescent stereomicroscopy and new fragments were dissected for serial engraftment.

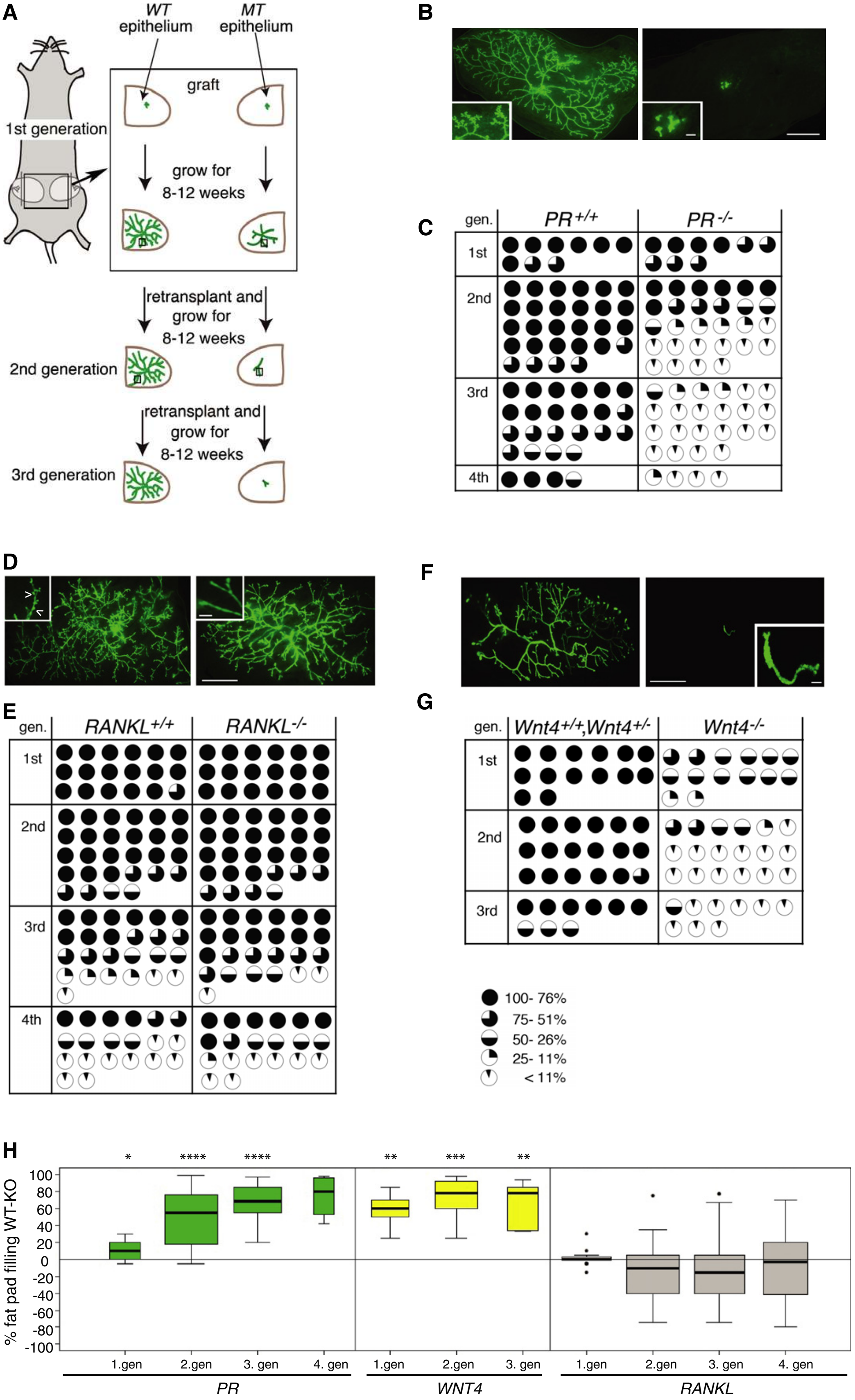
B, C Serial transplantations of $PR^{+/+}$ and $PR^{-/-}$ mammary epithelia. (B) Fluorescence stereo micrographs of third-generation mammary outgrowths derived from 8-week-old $PR^{+/+}$; *EGFP* and $PR^{-/-}$; *EGFP* donor mice. (C) Table summarizing 3 independent serial transplant experiments with $PR^{+/+}$; *EGFP* and $PR^{-/-}$; *EGFP*. Each engrafted gland is represented by a micrograph; black sectors represent area of fat pad filled by engrafted epithelium. Scale bar: 200 μm.

D, E Serial transplantation of $RANKL^{+/+}$ and $RANKL^{-/-}$ mammary epithelia. (D) Fluorescence stereo micrographs of third-generation mammary outgrowths derived from 5-week-old $RANKL^{+/+}$; *EGFP* and $RANKL^{-/-}$; *EGFP* donor mice. Insets: higher magnification showing side branches present in the WT control (arrowheads) absent from $RANKL^{-/-}$; *EGFP* epithelium. (E) Table summarizing three independent serial transplant experiments with $RANKL^{+/+}$; *EGFP* and $RANKL^{-/-}$; *EGFP* donor mice. Scale bar: 200 μm.

F, G Serial transplantation of $Wnt4^{+/+}$ and $Wnt4^{-/-}$ mammary epithelia. (F) Fluorescence stereo micrographs of third-generation mammary outgrowths derived from mammary buds of E12.5 and E13.5 $Wnt4^{+/+}$; *EGFP* and $Wnt4^{-/-}$; *EGFP* embryos. Scale bar: 200 μm. (G) Table summarizing three independent serial transplant experiments with $Wnt4^{+/+}$; *EGFP* donor mice. Scale bar: 200 μm.

H Box plot showing the difference between percentage of reconstitution between WT and MT contralateral grafts in each transplant generation. *P* values were determined by Mann–Whitney *U*-test.

▶


A
WT epithelium
MT epithelium
graft
1st generation
grow for 8-12 weeks
retransplant and grow for 8-12 weeks
2nd generation
retransplant and grow for 8-12 weeks
3rd generation
B
C
gen.
PR+/+
PR-/-
1st
2nd
3rd
4th
D
E
gen.
RANKL+/+
RANKL-/-
1st
2nd
3rd
4th
F
G
gen.
Wnt4+/+,Wnt4+/-
Wnt4-/-
1st
2nd
3rd
100- 76%
75- 51%
50- 26%
25- 11%
< 11%
H
% fat pad filling WT-KO
100
80
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PR
WNT4
RANKL

Figure 1.

does not appear to be essential in the control of the mammary stem cells under physiological conditions.

Control of Wnt4 expression

Previous studies suggested that Wnt4 is expressed only concomitant with high progesterone secretion in the adult and pregnant mammary gland (Gavin & McMahon, 1992; Weber-Hall *et al*, 1994). The finding that deletion of Wnt4 affected mammary regeneration potential more severely than abrogation of PR signaling suggested that Wnt4 may have PR-independent functions in stem cell stimulation. To detect Wnt4 expression at low levels, we crossed mice which express Cre from the Wnt4 locus (*Wnt4::Cre)* (Shan *et al*, 2009) to the *mT/mG* dual Cre reporter strain in which ubiquitous tomato expression is replaced by membrane EGFP upon Cre activation (Muzumdar *et al*, 2007). We failed to detect expression in embryos (E12.5 and E18.5) and were unable to detect EGFP in the mammary glands of newborn or 3-day-old double transgenic mice (*Wnt4::GFP)*. EGFP expression was detected on postnatal day 5 by fluorescence stereomicroscopy (Fig 2A) prior to the onset of ovarian function. Immunofluorescence of histological sections revealed EGFP expression in scattered mammary epithelial cells on day 5 (Fig 2B) and day 10 (Fig 2C). PR expression was not detected at these stages (Fig 2B and C). Consistent with Wnt4 being a PR target, the double immunofluorescence for histological sections from postnatal double transgenic mice (*Wnt4::EGFP)* suggested that EGFP expression is restricted to PR^{+} luminal cells in the glands of pubertal mice (Fig 2D), adult (Fig 2E and inset), and pregnant females (Fig 2F and inset). To assess whether myoepithelial cells may express EGFP, we performed triple immunofluorescence for EGFP, PR, and the myoepithelial marker p63. In mammary epithelia from 5-day-old *Wnt4::EGFP* females ($n = 7$), rare double positive cells were detected, and in most sections, cells expressed either p63 or EGFP (Fig 2G). In 4- (Fig 2H) and 8-week-old (Fig 2I) females, p63 and EGFP staining labeled distinct cells. Thus, Wnt4 is expressed almost exclusively in luminal cells. The Wnt4 expressing cells appear to be terminally differentiated as no clonal clusters of EGFP^{+} cells are observed.

To assess whether trace amounts of estrogens and progesterone of maternal origin could account for this perinatal Wnt4 expression, we analyzed d15 mammary glands from the *Wnt4::EGFP* mice on *WT*, $ER\alpha^{-/-}$, and $PR^{-/-}$ genetic backgrounds by epifluorescence stereomicroscopy. At this stage, ductal outgrowth was comparable by red fluorescence (Fig 2J–L). Neither *ERα* nor *PR* deletion altered *Wnt4::EGFP* expression (Fig 2M–O) indicating that perinatal Wnt4 expression is largely independent of ERα and PR signaling.

To determine the respective roles of the two major ovarian hormones in control of Wnt4 expression, we pooled epithelial-enriched organoids freshly isolated from mammary glands of pubertal and adult females ($n = 3$) and stimulated them for 6 h *ex vivo* (Fig 2P) (Ayyanan *et al*, 2011). Progesterone induced *Wnt4* mRNA expression in pubertal and adult organoids to 8.7- and 4.5-fold, respectively, whereas E2 elicited a 1.6-fold induction of *Wnt4* mRNA in the pubertal organoids only (Fig 2Q). To assess the physiological importance of PR signaling for pubertal Wnt4 expression, we grafted *Wnt4::GFP* epithelium derived from donors either $PR^{-/-}$ or *PRWT* to contralateral cleared fat pads of 3-week-old hosts. The engrafted glands were analyzed 3 weeks later when the recipients were pubertal. Epifluorescence stereomicroscopy for dTomato revealing unrecombined cells confirmed the presence of ductal outgrowth of *PRWT* and $PR^{-/-}$ grafts (Fig 2R and S). EGFP expression was readily detected in the *PRWT* graft (Fig 2T) but completely absent from some of the $PR^{-/-}$ grafts (Fig 2U). Double epifluorescence stereomicroscopy on a *PRWT* control graft reveals that EGFP is strongly enriched in the TEBs (Fig 2V and X), and in the contralateral $PR^{-/-}$ grafts, some EGFP expression is observed at the origin of the outgrowth (Fig 2W). These findings are consistent with perinatal ER-/PR-independent Wnt4 expression and indicate that pubertal Wnt4 induction is mediated by PR signaling.

Consequences of Wnt4 ablation on cell proliferation

The observation that Wnt4 deletion impaired the regenerative capacity of the mammary epithelium more severely than PR deletion did, pointed to a role of Wnt4 before puberty. The fat pad grafting approach used to determine Wnt4 function (Brisken *et al*, 2000) assesses gene function from puberty onward because the donor epithelium is placed into a 3-week-old host. To determine the role of Wnt4 perinatally and at the onset of puberty, we conditionally

Figure 2. Control of Wnt4 expression.

A Epifluorescence stereo micrograph of inguinal mammary gland from a 5-day-old *Wnt4::Cre; mT/mG* female ($n = 7$). Scale bars: 0.5 mm and 0.1 mm (inset).

B, C Histological sections of mammary glands from a 5-day-old (B; $n = 7$) and a 10-day-old (C; $n = 5$) *Wnt4::Cre; mT/mG* female stained by double immunofluorescence for EGFP (green) and PR (magenta, not detected), counterstained with DAPI (blue). Scale bar: 50 mm.

D–F EGFP (green) and PR (magenta) co-immunofluorescence counterstained with DAPI (blue) on histological sections from *mT/mG; Wnt4::Cre* mammary glands at different developmental stages. (D) TEB of a 4-week-old female ($n = 4$); scale bar: 30 μm. (E) Ducts of an 8-week-old female ($n = 3$); scale bar: 100 μm; inset, scale bar: 20 μm. (F) Duct of a female at day 10.5 of pregnancy ($n = 3$); scale bar: 150 μm; inset, scale bar: 30 μm.

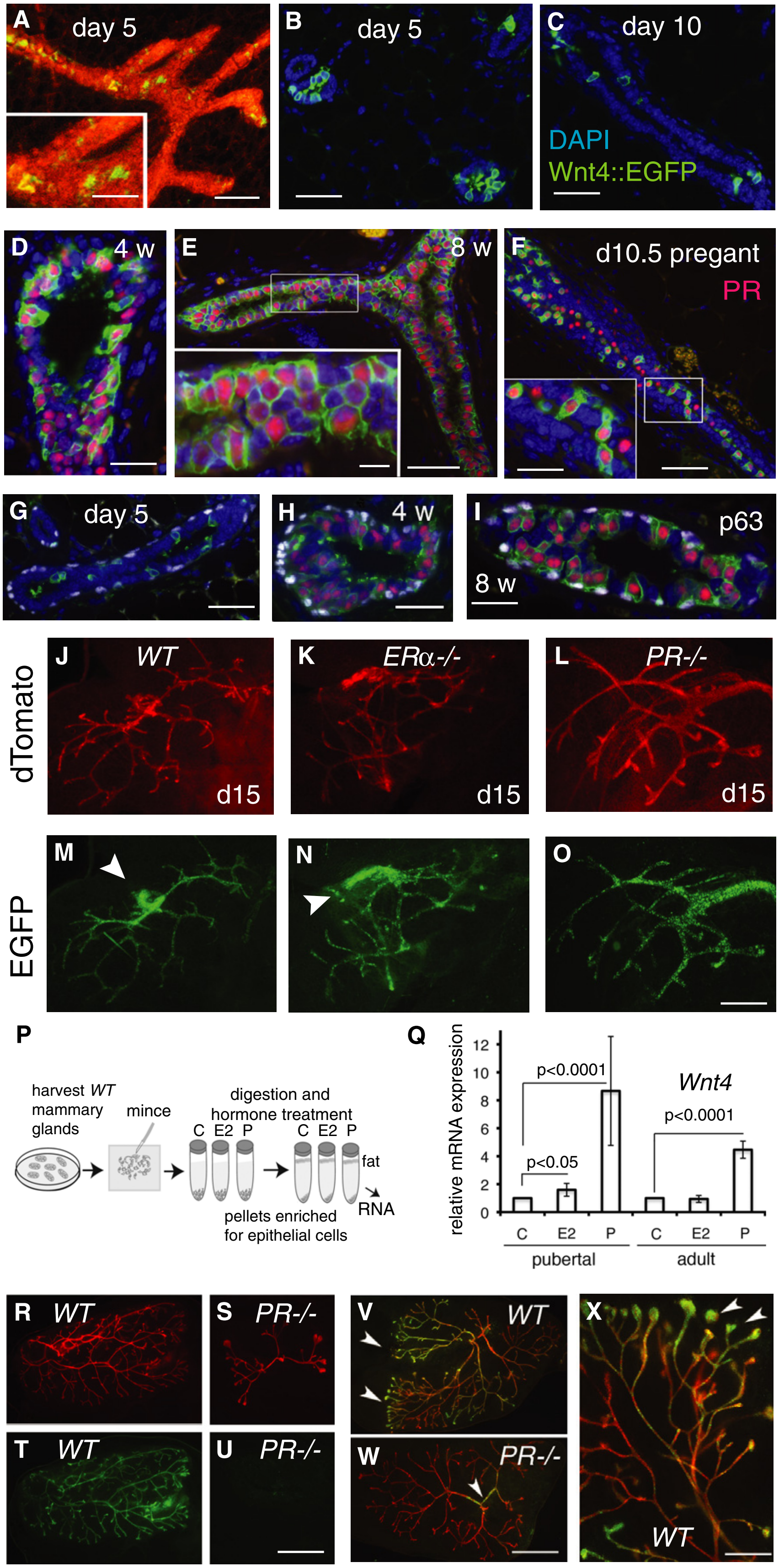
G–I EGFP (green), PR (magenta), and p63 (white) triple co-immunofluorescence counterstained with DAPI (blue) on histological sections from *mT/mG; Wnt4::Cre* mammary glands at different developmental stages. (G) Ducts of 5-day-old female ($n = 3$). (H) TEB of a 4-week-old female ($n = 3$). (I) Duct of an 8-week-old female ($n = 3$). Scale bars: 30 μm.

J–O Epifluorescence stereo micrographs of mammary glands harvested from 15-day-old *Wnt4::GFP* females either WT ($n = 18$) (J, M), $ER\alpha^{-/-}$ ($n = 4$) (K, N), or $PR^{-/-}$ ($n = 3$) (L, O). dTomato expression (J–L); EGFP expression (M–O) is not abrogated in $ER\alpha^{-/-}$ nor $PR^{-/-}$ epithelia. Arrowheads mark the main duct originating from the nipple. Scale bar: 50 μm.

P Scheme of *ex vivo* hormone stimulation of mammary organoids.

Q Bar plots showing relative *PR* and *Wnt4* mRNA expression normalized to *CK18* mRNA in mammary organoids from 5 pubertal (6 weeks old) and 3 adult (11 weeks old) mice exposed for 6 h to vehicle (C), 17β-estradiol (20 nmol) (E2), or R5020 (20 nmol) (P). Bars represent the mean ± SD of 3 independent experiments.

R–X Epifluorescence stereo micrographs of contralateral mammary glands that were engrafted with *Wnt4::GFP* epithelium from 8-week-old females, either *PRWT* (R, T, V, X) or $PR^{-/-}$ (S, U, W). dTomato expression (R, S); EGFP expression (T, U) double epifluorescence (V, W, X) on contralateral engrafted glands 3 weeks after surgery when recipients were 6 weeks old. Representative result from three independent experiments. Arrowheads point to TEBs (V, X) or to origin of growth (W). Scale bar (R–W): 5 mm, (X): 1 mm.


A
day 5
B
day 5
C
day 10
DAPI
Wnt4::EGFP
D
4 w
E
8 w
F
d10.5 pregant
PR
G
day 5
H
4 w
I
p63
8 w
dTomato
J
WT
d15
K
ERα-/-
d15
L
PR-/-
d15
EGFP
M
N
O
P
harvest WT mammary glands
mince
digestion and hormone treatment
C E2 P
C E2 P
fat
RNA
pellets enriched for epithelial cells
Q
relative mRNA expression
0
2
4
6
8
10
12
p<0.0001
p<0.05
Wnt4
p<0.0001
C
E2
P
C
E2
P
pubertal
adult
R
WT
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PR-/-
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WT
X
T
WT
U
PR-/-
W
PR-/-
WT

Figure 2.

deleted Wnt4 in the mammary epithelium by crossing mice with two conditional Wnt4 alleles (*Wnt4*$^{fl/fl}$) (Shan *et al*, 2010) to mice that express Cre in the mammary epithelium under the control of the MMTV-LTR (A-strain) (Wagner *et al*, 2001). To identify cells in which Cre-mediated recombination had occurred, the mice were crossed to the *mT/mG* dual Cre reporter strain (Muzumdar *et al*, 2007). Analysis of *MMTV::Cre; mT/mG* double transgenic females revealed widespread EGFP expression at postnatal day 10 both by stereo microscopy and (Fig 3A–E) immunofluorescence for EGFP (Fig 3F). In the *Wnt4* depleted *(MT)* mammary glands, a 10% decrease in the number of branching points was observed compared to control littermates around day 10 (Fig 3G and H). In pubertal *MT* glands, the number of terminal end buds (TEBs) had decreased to 54% of the controls (Fig 3I and J). Similarly, the area of fat pad filled by ducts was 60% of that measured in littermates (Fig 3I and K). Cell proliferation, as assessed by BrdU incorporation, was reduced to 65% of that in the *WT* counterparts in TEBs of Wnt4 mutants (Fig 3M). The proliferative index of about 6% in the subtending ducts was not affected in the Wnt4-deficient glands (Fig 3L). Thus, Wnt4 is required for perinatal and pubertal ductal expansion.

Activation of myoepithelial cells through canonical Wnt signaling

Wnt4 can activate its signaling, both canonical and non-canonical Wnt signaling (Lyons *et al*, 2004; Heinonen *et al*, 2011). Canonical Wnt signaling activity can be assessed *in vivo* using the *Axin2::LacZ* reporter mouse strain (Leung *et al*, 2002; Lustig *et al*, 2002) and was reported in a subset of CD29f^{hi} or CD49^{hi} breast stem cells

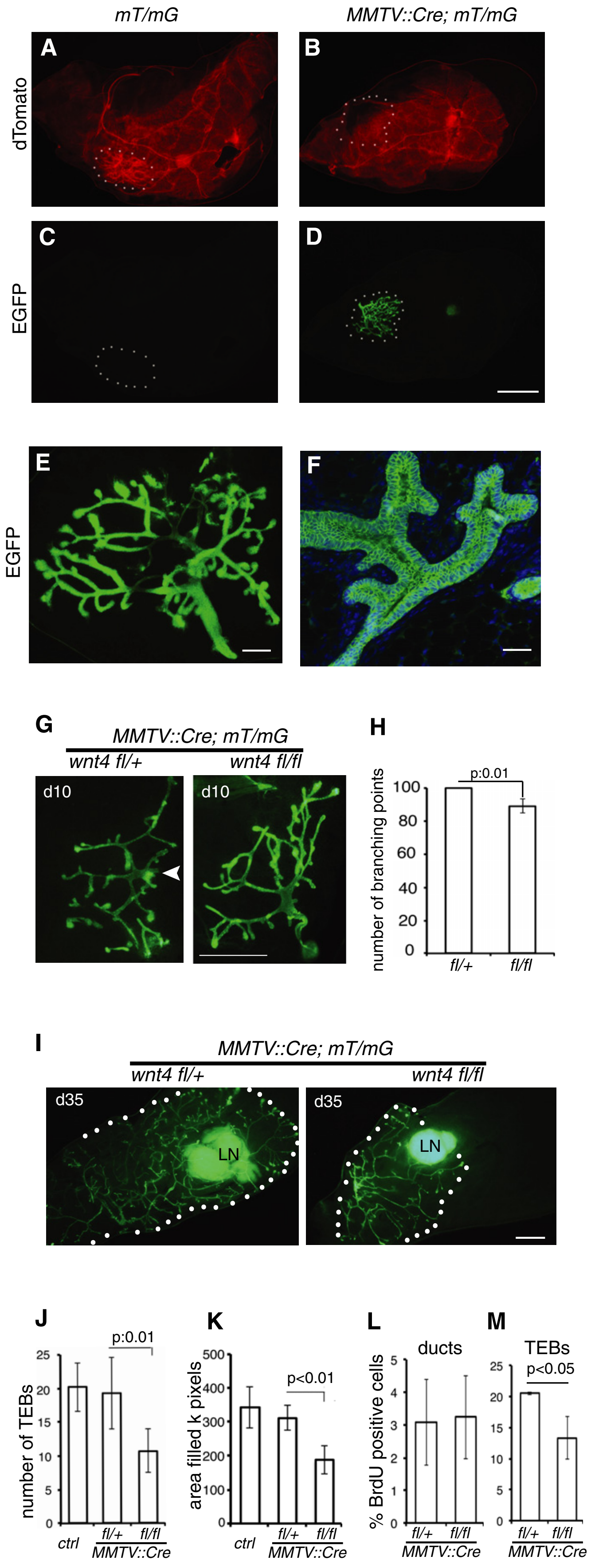

Figure 3. Conditional deletion of Wnt4 in the mammary epithelium and its consequences.

A–D Representative epifluorescence stereo micrographs of inguinal mammary glands from a 10-day-old *mT/mG* (A, C) and *MMTV::Cre; mT/mG* (B, D) female ($n = 7$). Scale bar: 1 mm.

E Higher magnification of inguinal mammary gland from a 10-day-old *MMTV::Cre; mT/mG* female. Scale bar: 0.2 mm.

F Immunofluorescence for EGFP (green) on a mammary gland section from a 10-day-old *MMTV::Cre; mT/mG* female counterstained with DAPI (blue) ($n = 7$). Scale bar: 30 μm.

G Epifluorescence stereo micrographs of mammary glands from 10-day-old littermates either *MMTV::Cre; mT/mG Wnt4*$^{fl/+}$ or *MMTV::Cre; mT/mG; Wnt4*$^{fl/fl}$. Scale bar: 1 mm. Arrowhead marks the main duct originating from the nipple.

H Bar plot showing ratio of branching points in prepubertal *Cre*$^{+}$;*Wnt4*$^{fl/fl}$ ($n = 12$) relative to *Cre*$^{+}$;*Wnt4*$^{fl/+}$ littermates ($n = 10$). Two-tailed, paired Student's *t*-test was used to calculate statistical significance.

I Fluorescence stereo micrographs of mammary glands from 35-day-old littermates either *MMTV::Cre; mT/m; Wnt4*$^{fl/+}$ or *MMTV::Cre; mT/m;Wnt4*$^{fl/fl}$. Note ductal elongation is delayed in *Wnt4*-deficient mammary epithelium compared to *Wnt4*$^{fl/+}$ control. LN: lymph node. Scale bar: 4 mm.

J, K Bar plots showing number of terminal end buds (TEBs) (J) and area of mammary fat pad filled by the ductal system (K). Ctrl (*Wnt4*$^{fl/wt}$ or *Wnt4*$^{fl/fl}$; *Cre*$^{-}$): $n = 6$, *fl/+*; *Cre*$^{+}$: $n = 4$, and *fl/fl*; *Cre*$^{+}$: $n = 7$). Data are presented as the mean ± SD. Two-tailed Student's *t*-test was used to calculate statistical significance.

L, M Bar plots showing BrdU incorporation index in inguinal mammary glands of 4-week-old *Wnt4*$^{fl/+}$; *Cre*$^{+}$ ($n = 3$) or *Wnt4*$^{fl/fl}$; *Cre*$^{+}$ ($n = 4$) in the ducts (L) and in the TEBs (M). Data are presented as the mean ± SD. Two-tailed Student's *t*-test was used to calculate statistical significance.

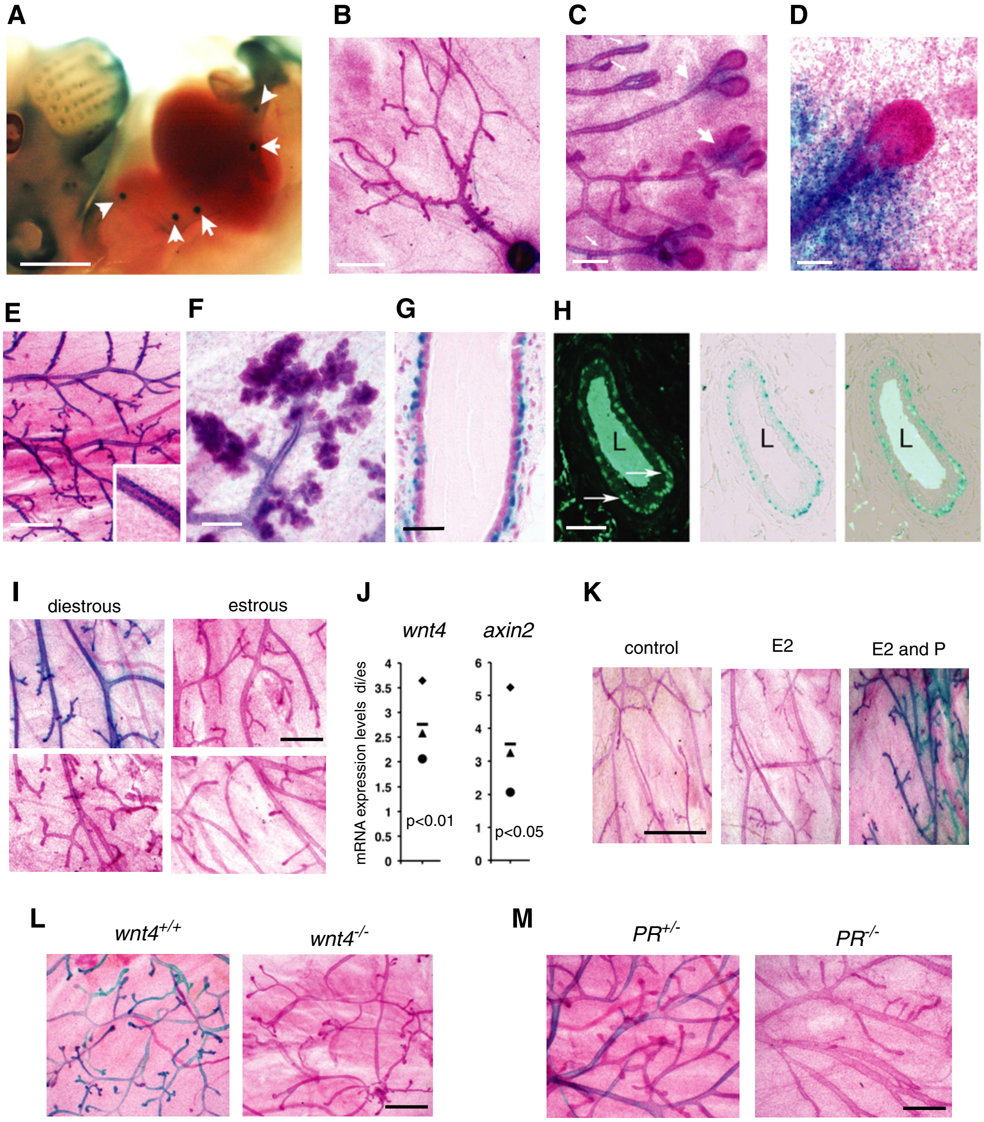

Figure 4. Canonical Wnt signaling activity during mammary gland development.

A Whole-mount micrograph of X-gal-stained *Axin2*$^{+/lacZ}$ in E12.5 embryo showing β-galactosidase expression in the mammary buds (arrows) (n = 8). Scale bar: 1 mm. Arrowheads mark mammary buds.

B–F Whole-mount micrographs of X-gal (blue)- and carmine alum (red)-stained mammary glands harvested from *Axin2*$^{+/lacZ}$ mice at distinct developmental stages. (B) At postnatal day 1, β-galactosidase activity detected in the nipple area (n = 6). Scale bar: 1 mm. (C, D) In 5-week-old mammary glands, reporter activity was detected around the ducts (small arrows) and in the neck region of the terminal end buds (TEBs) (large arrows) (C) (n = 8). Scale bars: 400 μm (C) and 100 μm (D). (E) At 8.5 day of pregnancy, reporter expression was detected in the ducts. Higher magnification (inset) suggests myoepithelial expression (n = 10). Scale bar: 1 mm. (F) Whole-mount at day 14.5 of pregnancy: reporter activity is limited to ducts (n = 5). Scale bar: 200 μm.

G Histological section of *Axin2::LacZ* mammary gland at day 8.5 of pregnancy counterstained with nuclear red; luminal epithelial cells show no detectable β-galactosidase activity but myoepithelial cells do. Scale bar: 200 μm.

H β-galactosidase activity (blue) colocalizes with the myoepithelial marker p63 (green) detected by immunofluorescence. Arrows point to myoepithelial cells. L, lumen. Scale bar: 100 μm.

I Representative whole-mount stereo micrographs of X-gal (blue)- and carmine alum (magenta)-stained mammary gland biopsies taken from 14-week-old *Axin2::LacZ* females collected at diestrus and estrus, respectively (n = 3). Scale bar: 200 μm.

J Relative *Wnt4* and *Axin2* mRNA expression in mammary glands from three mice in estrus versus diestrus assessed by semiquantitative qRT–PCR normalized to 18S rRNA. Two-tailed, paired Student's t-test was used to calculate statistical significance.

K Stereo micrographs of X-gal- and carmine alum-stained mammary glands from ovariectomized *Axin2::LacZ* females treated for 72 h with vehicle (n = 4) (left), 17-β-estradiol (E2) (n = 6) (center), 17-β-estradiol and progesterone (E2 and P) (n = 8) (right). Scale bar: 200 μm.

L, M Stereo micrographs of contralateral glands whole-mounted and X-gal stained after engraftment with mammary buds from *Axin2::LacZ* transgenic and *Wnt4*$^{+/+}$ or *Wnt4*$^{-/-}$ female E12.5 and E13.5 embryos (L) or *Axin2::LacZ* transgenic and either *PR*$^{+/-}$ or *PR*$^{-/-}$ 8-week-old females (M), at day 8.5 of pregnancy. β-galactosidase expression reflecting Axin2 transcription is readily detected in *PR*$^{+/-}$ as well as *Wnt4*$^{+/+}$ mammary epithelia but not in the *PR*$^{-/-}$ (n = 6) and *Wnt4*$^{-/-}$ counterparts (n = 6). Blue: X-gal staining; magenta: carmine alum counterstain. Scale bars: 200 μm.

(Zeng & Nusse, 2010; van Amerongen *et al*, 2012). We detected reporter activity in the mammary buds of E12.5/13.5 embryos (Fig 4A) consistent with previous reports based on an artificial Wnt signaling reporter (Chu *et al*, 2004). Perinatal β-galactosidase activity was confined to the nipple area (Fig 4B). Importantly, during puberty, LacZ expression was detected in the stroma surrounding the TEB necks (Fig 4C and D). In adulthood, β-galactosidase activity was readily detected in the ducts where it peaked on day 8.5 of pregnancy (Fig 4E). At day 14.5 of pregnancy, β-galactosidase activity was still detected in the ducts but not in the newly formed alveoli (Fig 4F). Histological sectioning suggested that X-gal staining mapped to the myoepithelial layer (Fig 4G); immunostaining with the myoepithelial marker p63 (Fig 4H) confirmed that LacZ is exclusively expressed in myoepithelial cells. Thus, canonical Wnt signaling in the postnatal mammary epithelium is confined to myoepithelial cells.

The peak in myoepithelial β-galactosidase activity during mid-pregnancy suggested that serum progesterone levels and hence Wnt4 expression may control canonical Wnt signaling activation. Indeed, when mammary glands in individual mice were analyzed, during progesterone-low estrous, they had lower β-galactosidase activity (Fig 4I) and lower *Wnt4* and *Axin2* mRNA levels than the glands analyzed during progesterone-high diestrous (Fig 4J); the fold differences varied between different animals (Fig 4I and J). To assess whether PR signaling induces canonical Wnt signaling, ovariectomized *Axin2::LacZ* females were treated with E2, to restore PR expression, and progesterone. Axin2 transcription reflected by β-galactosidase activity was induced by this combination but not by solvent or E2 alone (Fig 4K). Thus, progesterone stimulation results in increased transcription of Axin2, in the context of ER-dependent PR induction.

Multiple Wnts, some of which are secreted by mammary stromal cells, have been implicated in canonical Wnt signaling activation (Kessenbrock *et al*, 2013). To assess whether canonical Wnt signaling requires Wnt4 expression, we generated *Axin2::LacZ* transgenic mice in a $Wnt4^{-/-}$ background. In females engrafted with mammary buds from $Axin2::LacZ^{+}$ female embryos, β-galactosidase activity was readily detected in $Wnt4^{+/+}$ grafts at pregnancy day 8.5 but abrogated in the contralateral $Wnt4^{-/-}$ epithelia (Fig 4L). Similarly, it was decreased in $PR^{+/-}$ and abrogated in $PR^{-/-}$ epithelia (Fig 4M), indicating that both PR and Wnt4 are required for canonical Wnt signaling activation in the myoepithelium.

Our finding that Wnt4 expression is detected only from postnatal day 5 onward suggested that other family members, possibly Wnt10b, might be responsible for canonical Wnt signaling activation in the embryonic mammary bud. In line with this scenario, β-galactosidase activity was readily detected in the embryonic mammary buds of $Wnt4^{-/-}$*::axin2::LacZ* female E14.5 embryos (Fig 5A). To test whether the stromal Axin2 expression around TEB necks depends on epithelial Wnt4 expression, we generated *MMTV::Cre; axin2::LacZ* females either $Wnt4^{fl/fl}$ or $Wnt4^{fl/wt}$. Analysis of their mammary glands during puberty (6 weeks) showed that β-galactosidase activity was comparable between the two genotypes (Fig 5B). Thus, canonical Wnt signaling activation in the mammary bud and stroma is Wnt4 independent, whereas specifically in the myoepithelium, canonical Wnt signaling activation requires epithelial Wnt4 expression.

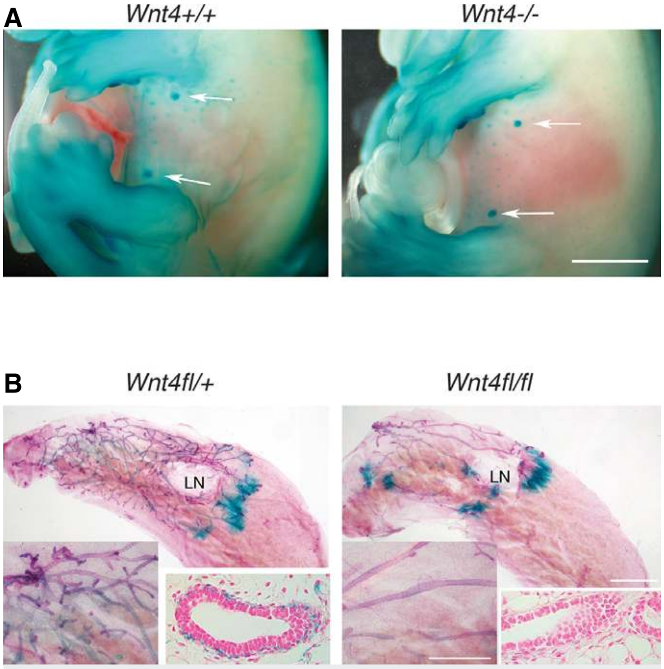

Figure 5. Wnt4-independent canonical Wnt signaling during mammary gland development.

A X-gal-stained whole-mounts of *Axin2::LacZ* embryos at E14.5 either $Wnt4^{+/+}$ or $Wnt4^{-/-}$ (n = 5 or 4, respectively). Arrows mark mammary buds. Scale bar: 5 mm.

B Whole-mount analysis of X-gal- and carmine alum-stained mammary glands harvested from 6-week-old littermates either $Wnt4^{fl/wt}$;$MMTV::Cre^{+}$; $Axin2^{+/lacZ}$ (n = 5) or $Wnt4^{fl/fl}$ $MMTV::Cre^{+}$;$Axin2^{+/lacZ}$ (n = 4). Left insets: higher magnification; right insets: histological section of the same gland. Note expression of reporter detected around the neck of TEBs in Wnt4-deficient and littermate control is comparable. LN, lymph node. Scale bars: 1 mm, and inset scale bar: 100 μm.

Discussion

Our data point to Wnt4 as a pivotal control factor of stem cell function for postnatal mammary gland development. We have uncovered a novel role for Wnt4 in perinatal development and puberty with progesterone as its major endocrine control factor. Progesterone, colloquially named 'pregnancy hormone,' appears as a primordial systemic factor in the postnatal mammary gland. It is the major proliferative stimulus to the adult mammary epithelium (Beleut *et al*, 2010) and controls the regenerative potential of the mammary gland by activating stem/progenitor cells throughout hormone-dependent development. Surprisingly, while hormone stimulation experiments had shown that estrogens induce Wnt4 expression (Brisken *et al*, 2000; Cai *et al*, 2014), we find that genetic deletion of PR signaling completely abrogated Wnt4 expression during puberty. Yet, the two ovarian hormones remain intertwined in Wnt4 control with ERα signaling acting indirectly as an upstream regulator of PR expression (Haslam & Shyamala, 1979) (Fig 6A).

We had previously analyzed Wnt4 function in the mammary gland by grafting $Wnt4^{-/-}$ embryonic buds to cleared fat pads of pubertal mice and therefore failed to discern the prepubertal function of Wnt4 uncovered through the use of the conditional Wnt4

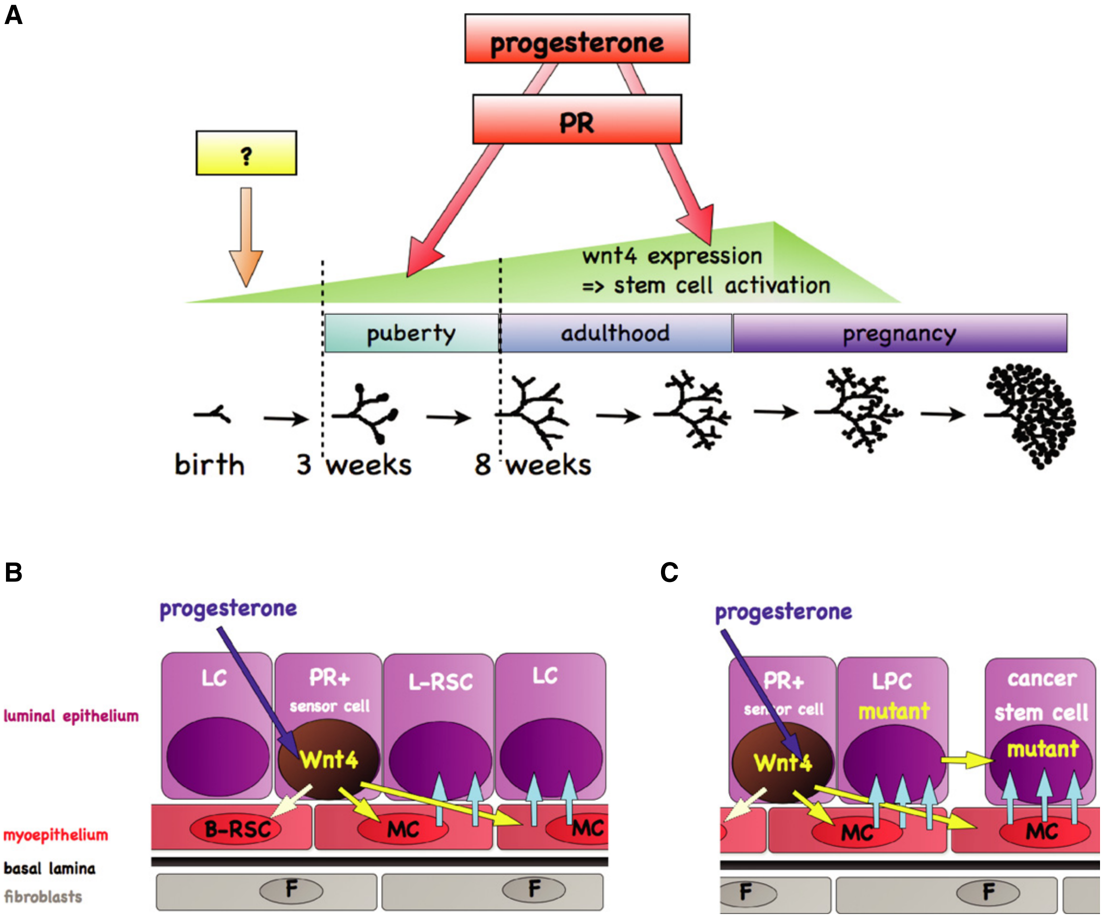

Figure 6. Models of hormonal stem cell control and Wnt4 action during mammary gland development.

A Control of mammary stem cell activity by Wnt4 during development. Schematic representation of mammary gland development (bottom) and control of mammary stem/progenitor cells by hormones (top). Model showing Wnt4 expression during mammary gland development. Wnt4 is important for stem cell activation throughout postnatal mammary gland development. Perinatal Wnt4 expression is independent of ER and PR, yet unknown factors control it. PR signaling is required for Wnt4 expression in puberty and adulthood. PR expression is induced by ER signaling.

B Model of Wnt4 action in the mammary epithelium. Progesterone stimulation results in Wnt4 induction in the PR^{+} luminal cells (LC), the 'sensor cells.' The secreted Wnt4 acts on adjacent basal/myoepithelial cells (MC). In the myoepithelial cells, Wnt4 activates canonical Wnt signaling which induces changes in gene expression. This results in the secretion of factors and changes in the ECM (light blue arrows) that in turn impinge on stem cells (SC), luminal-restricted stem cells (L-RSC), and basal-restricted stem cells (B-RSC). Wnt4 may also act directly on stem cells that are found within the basal layer.

C Model for the tumorigenic effects of progesterone and Wnt4 in the mammary epithelium. Repeated activation of this intercellular signaling cascade downstream of PR signaling may promote tumorigenesis by expanding luminal progenitor cells with oncogenic mutations and by expanding the stem/progenitor cell compartment.

Data information: In (B, C), black: basal lamina, F: fibroblast, LC: luminal cell.

allele in the present work. The ability of *Wnt4*$^{-/-}$ epithelium to form alveoli, which was preserved in previous transplants (Brisken *et al*, 2000), was also observed upon serial transplantation when mice were impregnated in this study.

Our finding that RANKL is not important for stem cell potential is in apparent contradiction with previous results based on assays with dissociated cells (Asselin-Labat *et al*, 2010). Compared to the widely used single-cell-based assays in which a defined number of cells are injected, grafting of intact epithelial fragments does not allow one to determine the number or fraction of cells endowed with regenerative potential. The approach does not disentangle the role of different types of stem and progenitor cells and their relative contributions to the outgrowth cannot be defined. Yet, the method is robust and multiple repeats of the experiment combined with several rounds of transplantation give a semiquantitative appreciation of an intact regeneration potential in a physiological tissue context that is missed by the use of dissociated cells. In fact, to our knowledge, this is currently the only stem cell assay in which at least part of the microenvironment remains intact that is so important to stem cell function. When single cells are injected into a cleared fat pad, they need to survive and to adhere to the stroma, an environment they are not exposed to physiologically. Both of these biological activities require integrin signaling and control each other through direct cadherin-mediated cell–cell contacts. In the widely used FACS-based stem cell assays, mammary stem cells are selected for high expression of integrin β1 or α6 (Shackleton *et al*, 2006; Stingl *et al*, 2006). It is conceivable that the selected cells are not intrinsically better 'stem cells' but that they have a strong advantage in adhering and surviving in the cleared fat pad, which is a *conditio sine qua non* for giving rise to *de novo* ducts. Alternatively, RANKL may be specifically required for the bipotential stem cells that are revealed under the challenging conditions of the single cell grafts. In the serial transplant approach, which is based on intact pieces of mammary tissue, the luminal- and the basal-restricted stem cells likely account for most of the cellular proliferation during development and a role of RANKL for the bipotent progenitors may not be discerned.

Our finding that Wnt4 secreted by PR^+ luminal cells activates canonical Wnt signaling exclusively in the neighboring basal/$p63^+$ cells, points to a scenario, in which the myoepithelial/basal cells are a central component of the microenvironment or 'niche' that controls different types of stem cells (Fig 6B). The myoepithelial/basal cells are ultimately under control of progesterone and Wnt4; hence, stem cell activity controlled through the microenvironment/niche is linked to reproductive needs. Whether the rare bipotent mammary stem cells that are found in the basal layer (Wang *et al*, 2014) are directly and/or indirectly activated by Wnt4 is not addressed by our experiments but will be an exciting line of future work.

The finding that the myoepithelial/basal cells are the prime target of Wnt4 bears on a long-standing conundrum. Wnt1 was long identified as an oncogene in the mouse mammary gland, and Wnt signaling is key for the development of the mammary gland. Yet, mutations in intracellular Wnt signaling components have not been found in breast carcinomas, which are of luminal origin. It is conceivable that Wnt signaling activation in the myoepithelial cells indirectly promotes tumorigenesis by inducing gene expression changes that result in the secretion of stimulatory signals and/or modulation of the extracellular matrix that result in the activation of luminal progenitor cells. Luminal progenitors with acquired oncogenic mutations could be expanded in response to Wnt4 stimulation of the myoepithelium (Fig 6C). In parallel, Wnt4, through its direct and/or indirect action on bipotent mammary stem cells, increases the number of stem cells. This in turn may result in an increased pool of luminal progenitors, which are more prone to oncogenic insults than more differentiated luminal cells.

These findings have clinical implications. The activation of the progesterone/Wnt4 pathway, which also operates in the human breast (Tanos *et al*, 2013; Pardo *et al*, 2014), may underlie the tumor promoting effects of recurrent menstrual cycles, oral contraception, and combined hormone replacement therapy with progestins. Selective progesterone receptor modulators and Wnt inhibitors, alone or together with RANKL inhibitors, may therefore be effective in breast cancer management, in particular, as preventive strategy in high-risk premenopausal women.

Materials and Methods

Animals

Axin2::LacZ (Lustig *et al*, 2002), *C57BL/6-Tg(Act-EGFP)* (Okabe *et al*, 1997), $ER\alpha^{-/-}$ (Dupont *et al*, 2000), *MMTV::Cre* (line A) (Wagner *et al*, 1997), *mT/mG* (Muzumdar *et al*, 2007), $PR^{-/-}$ (Lydon *et al*, 1995), $RAG1^{-/-}$ (Mombaerts *et al*, 1992), $RANKL^{-/-}$ (Wong *et al*, 1999), $Wnt4^{-/-}$ (Stark *et al*, 1994), $Wnt4^{Cre}$ (Shan *et al*, 2010), and $Wnt4^{fl/fl}$ mice (Shan *et al*, 2009) were kept on mixed genetic background 129SV/C57Bl6. All mice were maintained and handled according to the Swiss guidelines for animal safety. The ethic veterinary committee of canton of Vaud, Switzerland, approved all the animal experiments (Permit ID 1641.2 and 1641.3). To stage the estrus cycle, the vagina was flushed with 10 µl of PBS and vaginal secretions were collected, spread onto glass slides, and analyzed for different cell types (Caligioni, 2009). Mammary bud and mammary epithelial transplantations from 8-week-old donors were performed as described (Brisken *et al*, 2000). For serial transplantations, $EGFP^+$ mammary duct outgrowth was visualized by stereo epifluorescensce. Tissue fragments were prepared and retransplanted starting from at least three independent donors. Hormone treatments and BrdU injections were performed as described in Beleut *et al* (2010).

Mammary whole-mounts and image analysis

Mammary gland whole-mounting and (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) X-gal staining were performed as described (Brisken *et al*, 1998). Images were acquired either using a LEICA MZ FLIII stereomicroscope with PixeLINK (PL-A662) camera or LEICA M205FA/MZ16F fluorescent stereomicroscope with Leica DFC 340FX or Leica DC300F camera. Area of the fat pad filled by the mammary ducts was quantified by drawing a contour around the mammary ductal tips using Axiovision Rel 4.7 software. TEBs and branching points were quantified on images of whole-mounted mammary glands and on fluorescence stereo micrographs.

Immunostaining

Mammary glands were fixed in 4% paraformaldyde for 2 h at room temperature and embedded in paraffin. Five-micrometer sections were used for nuclear red and for immunohistochemical or immunofluorescence staining. Anti-p63 Molecular Probes 4A4 (1:100), anti-BrdU Oxford biotechnology, OBT0030, 1:300), anti-PR Thermo Fisher Scientific Pierce-MA1-411 (1:500), anti-GFP Molecular Probes A11122 (1:800). Images were acquired on LEICA DM 2000 microscope with a PixeLINK (PL-A662) camera or on Zeiss Axioplan 2-imaging fluorescence microscope with Axiocam MRm camera.

RNA extraction and semiquantitative RT–PCR

Mammary glands were homogenized in TRIzol (Invitrogen). Total RNA was isolated from fragments using RNeasy (Qiagen). cDNA was synthesized using random $p(dN)_6$ primers (Roche Diagnostics) and MMLV reverse transcriptase (Invitrogen). Semiquantitative real-time PCR analysis in triplicates was performed with SYBR Green PCR Core Reagents system (Qiagen)/PerfeCTa SYBR Green SuperMix for iQ™ (Quanta) on Realplex^2 (Eppendorf) or 7900HT Fast Real-Time PCR System (Applied Biosystems) qRT–PCR detection systems. All reactions were performed in triplicate. The following primers sequences were used: *Wnt4*, AGG AGT GCC AAT ACC AGT TCC, TGT GAG AAG GCT ACG CCA TA; *Axin-2*, GGC AGT GAT GGA GGA AAA TG, TGG GTG AGA GTT TGC ACT TG; *CK18*

(Schroeder & Lee, 1998); *18S rRNA*, GCA ATT ATT CCC CAT GAA CG, GGC CTC ACT AAA CCA TCC AA.

Statistics

Two-tailed, paired Student's *t*-test was used to calculate statistical significance; data are shown as means ± SD. Statistical analyses were carried out using Microsoft Excel. For serial transplantation, the extent of fat pad filling in percentage at each generation was compared by Wilcoxon signed rank test between contralateral outgrowths. The statistical software R was used for analysis. The statistical test used and *P*-values are indicated in each figure legend. $P < 0.05$ was considered to indicate statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

RRD and CB designed experiments. MC, AA, and OYO performed experiments. RRD and DB performed experiments and analyzed data. JR and CB analyzed data. JS and SV provided mouse models. SV and CB contributed in writing the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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