Cellular and molecular effects of early pregnancy on mammary epithelial cell subpopulations in mice and their potential relevance for breast cancer protection

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1. SUMMARY

The breast cancer protective effect of an early pregnancy is well established in both humans and rodents, but the underlying mechanism is unclear. Since breast cancers are thought to originate from distinct mammary epithelial cell subtypes, we studied the effect of early parity on the gene expression and the functional properties of specific mouse mammary epithelial cell subpopulations. The latter were isolated by fluorescence-activated cell sorting (FACS) from parous and from age-matched virgin control mice. The isolated cell subpopulations were investigated further by unbiased genomic and bioinformatic methods, as well as by *in vitro* colony formation and by *in vivo* mammary gland reconstitution assays. The results of the transcriptome analysis showed an upregulation of differentiation genes and a pronounced decrease in the Wnt/Notch signaling ratio in the basal stem/progenitor cell subpopulation of parous mice. This was associated with a parity-induced downregulation of carcinogenic pathways and a reduction in the *in vitro* and *in vivo* proliferation potential. As a possible mechanism for reduced Wnt signaling in basal stem/progenitor cells, we found a more than threefold decrease in the expression of the secreted Wnt ligand *Wnt4* in isolated total mammary cells from parous mice, which corresponded to a similar reduction in progesterone receptor positive and Wnt4-secreting cells in intact mammary epithelia. Notably, recombinant Wnt4 partially rescued the parity-induced *in vitro* proliferation defect of basal stem/progenitor cells, strongly suggesting a causal relation between decreased Wnt4 secretion and parity-induced molecular and functional changes of basal stem/progenitor cells in mice. In conclusion, the study shows that early parity induces differentiation, downregulates the Wnt/Notch signaling ratio and decreases the *in vitro* and *in vivo* proliferation potential of basal mammary stem/progenitor cells in mice. Thereby, the study not only delineates the cellular and molecular effects of early parity, but it also paves the way for future studies

examining whether inhibitors of Wnt signaling can be used to mimic the parity-induced protective effect against breast cancer.

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3. INTRODUCTION

Pregnancy is the most significant modifiable factor affecting a woman's risk to develop breast cancer. Whilst a transient increase in breast cancer risk is observed immediately after parturition in women over 25 years, the long-term consequences of pregnancy include a strong and life-long breast cancer protective effect (Albrektsen et al., 2005; MacMahon et al., 1970). Thereby, parity-induced tumor protection is more pronounced the earlier the pregnancy has occurred (MacMahon et al., 1970). Similarly, pregnancy and pregnancy-mimicking hormones decrease the lifetime risk of developing mammary tumors in rodents (Medina, 2005; Rajkumar et al., 2007). Although early parity has been known for decades to change the pathophysiology of mammary glands, the underlying mechanism has only recently started to be unraveled.

3.1 Mammary gland development and epithelial cell hierarchy

3.1.1 Mammary gland development

The mammary gland is unique in that it develops largely postnatally. Before puberty, the mammary gland contains only a rudimentary ductal system embedded in specialized stroma, known as the mammary fat pad in mice. With the onset of puberty (3 weeks in mice, 9-12 years in humans), a network of ducts starts to grow from special structures described as terminal end buds (TEBs) in mice and observed also in humans (Anbazhagan et al., 1998; Williams and Daniel, 1983) (see Figure 3.1.1). The ductal system continues to grow after sexual maturity (5 weeks in mice, 11-14 years in humans) and reaches its full dimensions at about 8 weeks of age in mice and 18-24 years in humans (Anderson et al., 2003; Brisken and Duss, 2007; Howard and Gusterson, 2000; Russo and Russo, 2011). The mature virgin mammary gland consists of an extensive ductal network and numerous budding structures, known as alveolar buds in mice and terminal ductal lobuloalveolar units (TDLUs) in humans

(Britt et al., 2007; Cardiff and Wellings, 1999; Smalley and Ashworth, 2003). During pregnancy, the second stage of postnatal mammary gland development, alveolar buds or TDLUs expand their ductal branches and differentiate into milk-producing structures termed lobular alveoli during lactation. As lactation ceases, the mammary gland regresses to a virgin-like state in a process called involution (Britt et al., 2007). This cycle of alveolar bud/TDLU expansion, differentiation into lobular alveoli and subsequent involution is repeated in following pregnancies. Notably, even though the involuted mammary gland resembles its virgin counterpart morphologically, it is not identical but retains vestiges of the preceding pregnancy (Cardiff and Wellings, 1999; Russo et al., 1982).

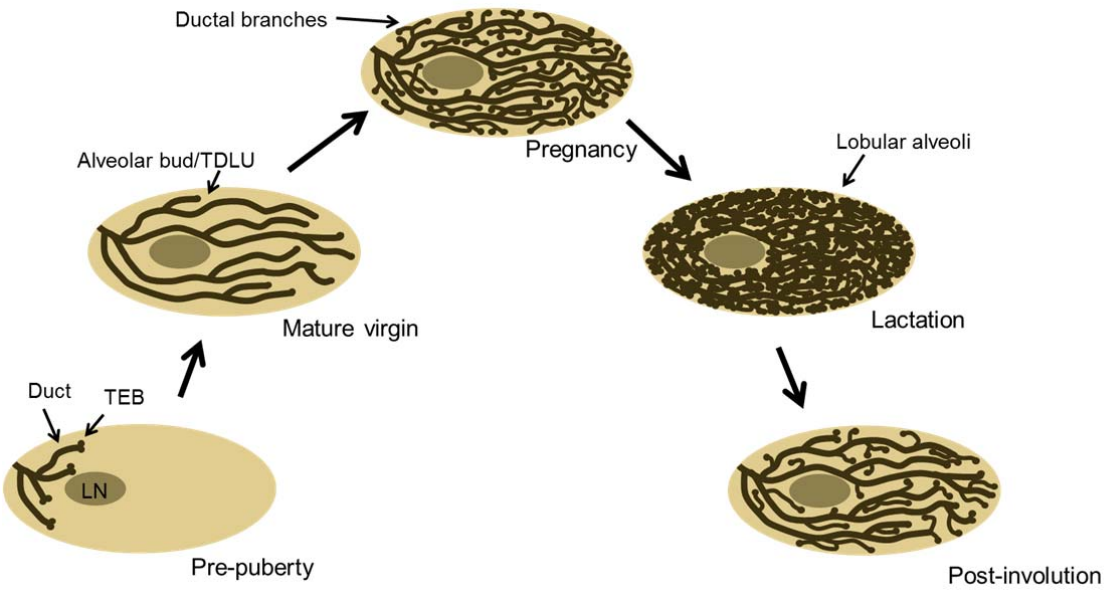

Figure 3.1.1 Mammary gland development

Schematic illustration of pre-pubertal and mature virgin mammary glands, as well as of mammary glands during pregnancy, lactation and after involution. TEB = terminal end bud, TDLU = terminal ductal lobuloalveolar unit, LN = lymph node

3.1.2 Cell hierarchy in the mammary gland epithelium

The mammary epithelium in humans and mice is hierarchically organized. It essentially comprises luminal and basal cell compartments, which are separated from the surrounding stromal tissue by a basement membrane (see Figure 3.1.2). Thereby, differentiated luminal and basal (myoepithelial) epithelial cells have to be distinguished from luminal and basal progenitor as well as from mammary stem cells (Bruno and Smith, 2011; Visvader, 2009). Differentiated luminal cells have a central role in milk production during the lactation period. Furthermore, they comprise estrogen and progesterone receptor positive cells and are responsible for mediating the effects of estrogen and progesterone to other mammary epithelial cell types (Brisken and Duss, 2007; Sleeman et al., 2007). Differentiated myoepithelial cells constitute the contractile units of the mammary gland required for milk ejection. Luminal and basal progenitor cells are precursors for differentiated luminal and basal myoepithelial cells. They arise from mammary stem cells and are characterized by their ability to form colonies *in vitro* (Smalley et al., 1998; Stingl et al., 2001; Stingl et al., 2006). Mammary stem cells are long-lived, have the capability to produce the other cell types when needed and give rise to new mammary epithelial tissue during puberty and pregnancy (Visvader, 2009). Traditionally, mammary stem cells are functionally defined by their potential to reconstitute complete mammary epithelium when transplanted into epithelium-free mouse mammary fat pads *in vivo* (Bruno and Smith, 2011; Deome et al., 1959). Such mammary stem cells, also known as mammary repopulating units (MRUs), are basally located and multipotent (able to form both luminal and basal epithelial cells) when transplanted into cleared fat pads (Shackleton et al., 2006; Sleeman et al., 2007; Stingl et al., 2006). In the context of the intact adult mammary gland, recent lineage tracing experiments suggest a unipotent nature of basal mammary stem cells and the existence of additional luminal mammary stem cells. In these studies, multipotent mammary stem cells were

observed solely in the embryonic and possibly in the pregnant gland (van Amerongen et al., 2012; Van Keymeulen et al., 2011). Mammary epithelial cells are separated by a basement membrane from the stromal tissue, which comprises fibroblasts, endothelial cells, macrophages, and adipocytes (Neville et al., 1998).

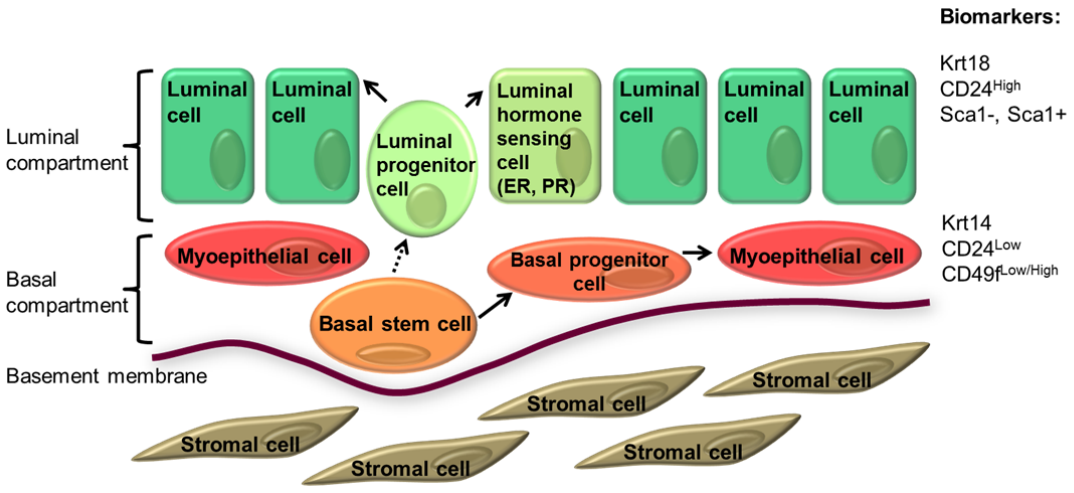

Figure 3.1.2 Mammary epithelial structure and cell hierarchy

Schematic cross-section through the mammary epithelium. Differentiated milk-producing luminal cells face the ductal lumen. They are closely associated with contractile basal myoepithelial cells, which serve in milk ejection. In the adult gland, differentiated luminal and basal myoepithelial cells develop from their corresponding progenitor cells. The entire epithelium is surrounded by a basement membrane and by stromal cells.

Distinct mammary epithelial cell subpopulations can be isolated by fluorescence-activated cell sorting (FACS) using specific cell surface markers both from human breast tissue and from mouse mammary glands (Raouf et al., 2012; Shackleton et al., 2006; Sleeman et al., 2007; Smalley et al., 2012; Stingl et al., 2006). In mice, the expression of heat stable antigen CD24, stem cell antigen1 (Sca1) and β1-integrin (CD29) or α6-integrin (CD49f), allows the

separation of luminal Sca1+ (CD24+^{High} Sca1+), luminal Sca1- (CD24+^{High} Sca1-), basal myoepithelial (CD24+^{Low} Sca1- CD49f^{Low}) and basal stem/progenitor (CD24+^{Low} Sca1- CD49f^{High}) cells. Luminal Sca1+ cells comprise estrogen receptor positive cells and display limited *in vitro* and no *in vivo* growth potential, suggesting a composition of many differentiated cells (Sleeman et al., 2007). Similarly, myoepithelial cells show little *in vitro* and *in vivo* growth capacity. They express smooth muscle actin (SMA) and are thought to represent basal differentiated cells (Smalley et al., 2012). In contrast, luminal Sca1- cells give rise to large and numerous colonies *in vitro* but have limited *in vivo* outgrowth capability, thereby featuring the classic phenotype of progenitor cells (Smalley et al., 2012; Smalley et al., 1998; Stingl et al., 2006). Finally, isolated CD49f^{High} basal stem/progenitor cells are highly enriched in mammary repopulating units (MRUs) and show strong *in vivo* and moderate *in vitro* growth potential (Shackleton et al., 2006; Sleeman et al., 2007; Stingl et al., 2006). These functional characteristics indicate a strong enrichment of basal mammary stem as well as progenitor cells in this cell subpopulation. The isolation of specific mammary epithelial cell subpopulations allows the study of cell subtype-specific properties and thus helps to understand intercellular communication in the mammary gland.

3.2 Signaling pathways important for postnatal mammary gland development

3.2.1 Hormones and Growth factors

Postnatal mammary gland development is under hormonal control with central roles for the ovarian hormones estrogen and progesterone, as well as the hypophyseal hormones growth hormone and prolactin. The hormones bind to receptors in specific mammary cells and elicit intra- and intercellular signaling cascades, which in turn stimulate developmental changes.

Estrogen

Ovarian estrogens are the major mitogenic factors for ductal morphogenesis (elongation/bifurcation) during puberty. Accordingly, ovariectomy in mice at 5 weeks of age causes failure of mammary ductal network development (Brisken and O'Malley, 2010; Neville et al., 2002), an effect which is rescued by implantation of 17-β-estradiol pellets into the mammary gland (Daniel et al., 1987). Mechanistically, estrogen appears to signal primarily via estrogen receptor alpha (ERα) to mediate ductal morphogenesis, and mice deficient in ERα exhibit severely stunted mammary development. Thereby, epithelial rather than stromal ERα expression is required, since ERα-/- epithelial cells fail to develop mammary ducts at puberty when transplanted into ERα wild type mammary fat pads, whereas ERα wild type epithelial cells form ductal networks in ERα-/- mammary fat pads (Bocchinfuso and Korach, 1997; Couse and Korach, 1999; Mallepell et al., 2006). Apart from estrogen itself, epidermal growth factor (EGF) also rescues ductal outgrowth in ovariectomized pubertal mice (Coleman et al., 1988), suggesting a downstream role of EGF-mediated signaling in the estrogen pathway. Amongst the EGF family members, only amphiregulin is transcriptionally upregulated by estrogen in luminal epithelial cells during puberty and was shown to be necessary for estrogen-mediated ductal morphogenesis in gene-knockout studies (Ciarloni et al., 2007). Following cleavage by ADAM17/TACE (Sunnarborg et al., 2002), estrogen-induced amphiregulin protein binds to and activates epidermal growth factor receptor (EGFR) expressed in the mammary gland stroma during ductal elongation (Schroeder and Lee, 1998) (see Figure 3.2.1a). Thereby, importance of stromal rather than epithelial EGFR is shown by transplantation experiments where EGFR-/- epithelium grafted into wild type fat pads produces normal ductal outgrowths, whereas wild type epithelium grafted into EGFR-/- stroma fails to develop ductal networks (Wiesen et al., 1999). EGFR signaling in stromal cells is thought in turn to cause the release of mitogenic

signals to mammary epithelial cells. Thereby, fibroblast growth factor (FGF) 2 and FGF7 are especially attractive candidates for downstream growth promoting stimuli (Hynes and Watson, 2010; Sternlicht et al., 2005). The final result of the estrogen-ERα-amphiregulin-EGFR-FGF signaling cascade is ductal elongation and bifurcation during puberty.

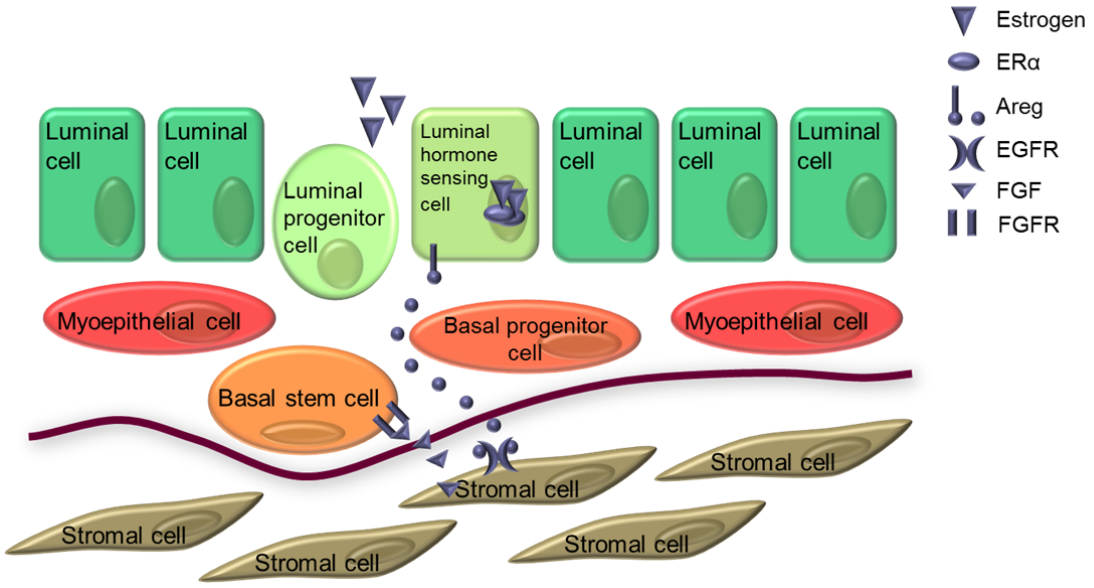

Figure 3.2.1a Estrogen signaling cascade

Estrogen binds to estrogen receptor alpha (ERα) in luminal epithelial cells and activates the expression of amphiregulin (Areg). Areg functions in paracrine signaling, binding to epidermal growth factor receptors (EGFRs) in stromal cells and inducing the stromal release of fibroblast growth factors (FGFs). Stromal FGFs are thought to stimulate basal stem cells to proliferate, ultimately leading to ductal growth (Pond et al., 2013). FGFR = fibroblast growth factor receptor

Growth hormone

Peripubertal ductal morphogenesis is dependent not only on estrogen but also on growth hormone (GH) and its downstream effector insulin-like growth factor-1 (IGF-1) (Neville et al., 2002). This is demonstrated by the failure of estrogen to rescue mammary development in hypophysectomized, ovariectomized rats and its ability to restore duct formation if GH or IGF-1 is co-administered (Kleinberg et al., 2000; Sternlicht, 2006). Thereby, paracrine rather than endocrine IGF-1 appears essential for mammary development, since global IGF-1 knockout mice exhibit significantly impaired duct formation whereas transgenic mice with liver-specific knockouts (resulting in 25% of wild type systemic IGF-1 levels) have normal ductal morphogenesis (Richards et al., 2004). Furthermore, IGF-1 receptor (IGF-1R)-deficient mammary epithelial transplants demonstrate reduced ductal outgrowths in wild type cleared fat pads, suggesting that epithelial rather than stromal IGF-1R is required for mammary development (Bonnette and Hadsell, 2001). In contrast, GH receptor is dispensable in the epithelium, indicating an important role for stromal GH receptor (Gallego et al., 2001). Taken together, the GH-IGF-1 axis is an essential accessory signaling pathway next to estrogen for pubertal ductal morphogenesis.

Progesterone

Estrus cycle and pregnancy-induced ductal side-branching and alveolar morphogenesis in mature mammary glands require progesterone signaling. In line with this, deletion of both progesterone receptor (PR) isoforms, PR-A and PR-B, leads to failure of tertiary side-branching and lobuloalveolar development in adult and pregnant mice whilst not affecting pubertal ductal morphogenesis (Soyal et al., 2002). Thereby, selective knockout experiments indicate that PR-B is essential and sufficient for these effects (Conneely et al., 2003), whereas tissue localization and transplantation studies support the importance of epithelial PR

expression for lobuloalveolar development and possibly stromal PR for ductal growth (Brisken et al., 1998; Humphreys et al., 1997). Two mediators of progesterone signaling are established in the mammary gland: the tumor necrosis factor (TNF) family member Receptor Activator for Nuclear Factor kappaB Ligand RANKL and the Wnt ligand Wnt4 (see Figure 3.2.1b). Both RANKL protein and Wnt4 co-localize with PR positive luminal epithelial cells adjacent to proliferating cells, and their expression is regulated by progesterone (Brisken et al., 2000; Mulac-Jericevic et al., 2003). Moreover, deletion of RANKL or Wnt4 results in impaired pregnancy-induced side-branching and alveogenesis (Brisken et al., 2000; Fata et al., 2000), whereas their ectopic expression causes tertiary side-branching in the absence of pregnancy (Bradbury et al., 1995; Fernandez-Valdivia et al., 2009). Thereby, Wnt4 is thought to act by stimulating canonical Wnt signaling and proliferation in basal stem and/or progenitor cells of adult virgin mice (Rajaram and Brisken, 2009). This is discussed in more detail below.

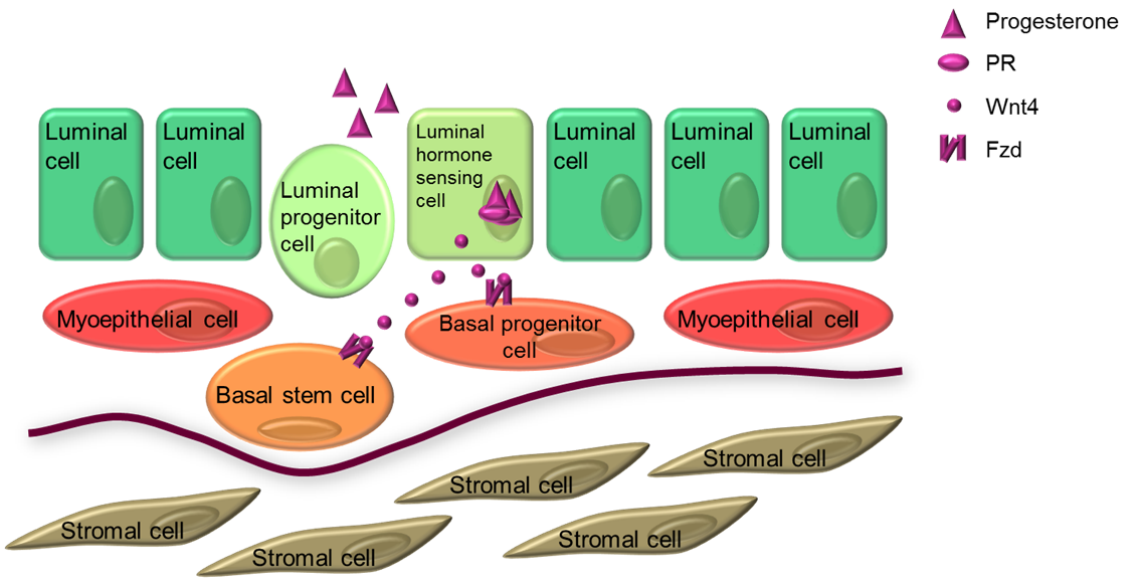

Figure 3.2.1b Progesterone signaling cascade

Progesterone binds to progesterone receptors (PRs) in luminal epithelial cells and activates the expression and secretion of the Wnt ligand Wnt4. Progesterone-stimulated Wnt4 acts on basal mammary stem and/or progenitor cells, promoting their proliferation and resulting in lobuloalveogenesis and tertiary side-branching. Fzd = Frizzled receptor

Prolactin

Final lactogenic differentiation of the mammary gland is under the control of prolactin (Neville et al., 2002). Transplantation experiments of prolactin receptor (PrlR) deficient epithelium demonstrate that prolactin signaling is required for alveolar development and lactogenesis during late pregnancy, but not for ductal outgrowth and side branching (Brisken et al., 1999). Thereby, epithelial rather than stromal PrlR expression appears central to mammary gland physiology, since wild type mammary epithelium transplanted into PrlR-/- stroma develops normally (Ormandy et al., 2003). Mechanistically, prolactin was shown to act via IGF-2 and cyclin D1 in the mammary gland (Brisken et al., 2002; Hovey et al., 2003).

In order to elicit growth responses in the mammary gland, the hormone- and growth factor-initiated signaling pathways must influence cell proliferation, lineage commitment and differentiation of specific mammary epithelial cell subtypes. The discovery of techniques to isolate and manipulate specific mammary epithelial cell subpopulations (see above) has greatly improved the understanding of the cell fate determining signaling pathways in postnatal mammary gland development and homeostasis.

3.2.2 Cell fate determining signaling pathways

Mammary gland growth in response to hormones/growth factors and tissue homeostasis relies on the ability of stem and progenitor cells to self-renew and/or to differentiate (Roarty and Rosen, 2010). Several signaling pathways have been found to govern these processes in mammary stem and progenitor cells, including Wnt, Notch, TGFβ, and receptor tyrosine kinase (RTK) signaling.

Wnt signaling

Wingless related protein (Wnt) signaling is central to cell fate decisions and stem cell homeostasis in many species and organs (Munoz-Descalzo et al., 2012). The Wnt proteins constitute a family of 19 highly conserved secreted glycoproteins. They initiate receptor-mediated signaling cascades, of which the Wnt/beta-catenin-dependent or 'canonical' pathway is best characterized (Angers and Moon, 2009). Canonical Wnt signaling involves the interaction of Wnt ligands with seven-pass-transmembrane-spanning Frizzled (Fzd) receptors and with co-receptors of the LDL-receptor-related protein family (Lrp5/6). Binding of Wnts to the receptor complex prevents destruction of β-catenin by a degradation complex containing glycogen synthase kinase-β (GSK-3β), axin, and adenomatous polyposis coli (APC). This allows β-catenin to accumulate in the nucleus and interact with the nuclear Lymphoid Enhancer Factor/T-Cell-Specific Transcription Factor (LEF/TCF) family of transcription factors, resulting in the expression of the respective target genes (Angers and Moon, 2009). Thereby, the identity of the target genes and thus also the effect on cell fate is tissue and cell type-specific (Hoppler and Kavanagh, 2007).

First indications for a major cell fate determining role of Wnt signaling in the mammary gland came from findings of differential expression patterns of Wnt ligands at specific

developmental stages (Gavin and McMahon, 1992). Of the Wnt proteins expressed in the mammary gland, specific roles have been defined for Wnt4 and Wnt5a. Whereas Wnt5a appears to regulate pubertal ductal morphogenesis (Roarty and Serra, 2007), Wnt4 acts downstream of progesterone, initiating tertiary side-branching during adulthood and early to mid-pregnancy (see above). Definitive evidence for a role of Wnt/β-catenin signaling in mammary epithelial cell fate decisions and self-renewal properties is provided by Wnt co-receptor Lrp5/6 knockout studies (Lindvall et al., 2006; Lindvall et al., 2009), administration of exogenous Wnt proteins to mammary basal stem/progenitor cells (Zeng and Nusse, 2010) and lineage tracing experiments for Wnt responsive mammary epithelial cells (van Amerongen et al., 2012): Lrp5 and Lrp6 deficient mammary glands are characterized by reduced side-branching (Lindvall et al., 2006; Lindvall et al., 2009), Lrp5/6 is expressed in the basal compartment harboring basal stem and progenitor cells, and Lrp5 expressing mammary cells have been demonstrated to exhibit self-renewing properties (Badders et al., 2009). Addition of purified Wnt3a protein to isolated mammary basal stem/progenitor cells increases their ability to clonally expand *in vitro* and maintains their mammary gland reconstitution efficiency upon transplantation *in vivo*. Furthermore, basal stem/progenitor cells mutant for the negative-feedback regulator Axin2 and thus sensitized to Wnt signaling show increased capacity to form functional glands in transplantation experiments (Zeng and Nusse, 2010). Moreover, tracing of Axin2 reporter expressing and Wnt/β-catenin responsive cells reveals that Wnt/β-catenin responsive mammary cells give rise to basal and luminal epithelial cells in a developmental stage-dependent fashion, further supporting the notion that Wnt/β-catenin signaling directs cell fate of mammary stem cells (van Amerongen et al., 2012).

Notch signaling

Similar to Wnt signaling, Notch signaling is involved in many cell fate decisions during animal development. The Notch gene encodes a transmembrane receptor with an intracellular part which is cleaved off upon specific ligand binding. In mammals, four Notch receptors (Notch 1-4) and five transmembrane ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4) have been identified and characterized (Borggrefe and Oswald, 2009). Upon cleavage, the Notch intracellular domain (NICD) translocates to the nucleus and binds the RBP-J transcription factor as well as mastermind-like (MAML) family members of co-activators (Petcherski and Kimble, 2000; Wu et al., 2000). The RBP-J/NICD/MAML complex then recruits histone acetyltransferase p300 to activate Notch target gene expression (Oswald et al., 2001; Saint Just Ribeiro et al., 2007; Wallberg et al., 2002).

In the mammary gland, the Notch pathway regulates stem and progenitor cell activity and commits mammary stem cells to the luminal cell lineage both in humans and mice (Bouras et al., 2008; Raouf et al., 2008). Accordingly, impaired Notch signaling in basal mammary stem/progenitor cells stimulates stem cell renewal and expansion, whereas constitutively active Notch specifically targets luminal progenitor cells for proliferation (Bouras et al., 2008). Similar studies in human bipotent progenitor cells underscore the conserved role of Notch in luminal cell fate commitment (Raouf et al., 2008). Interestingly, a prominent Notch target gene in the mammary gland is Gata3 (Bouras et al., 2008), which itself stimulates luminal cell differentiation (Asselin-Labat et al., 2007) and thus enforces and potentially even partially mediates Notch's effect on luminal commitment in the mammary gland.

TGFβ signaling

Transforming Growth Factor Beta (TGFβ) signaling has long been known to regulate cell proliferation, apoptosis, and differentiation during mammary gland development (McNally and Martin, 2011). Three isoforms of mammalian TGFβ exist, TGFβ1, TGFβ2 and TGFβ3, all of which bind the receptor TGFβRII which in turn recruits and activates Alk5/TGFβRI. Both TGFβRII and Alk5/TGFβRI are single-pass transmembrane serine threonine kinases and upon ligand binding, they phosphorylate downstream effectors, including SMAD members (Dunphy et al., 2011). Phosphorylated SMAD complexes subsequently move to the nucleus to regulate gene transcription. In the mammary epithelium, all isoforms of TGFβ are expressed at moderate levels in nulliparous mice with TGFβ2 and TGFβ3 being highly expressed during pregnancy and early involution (Faure et al., 2000; Nguyen and Pollard, 2000; Robinson et al., 1991). Experiments using slow-release pellets of TGFβ1 (Daniel et al., 1989; Silberstein and Daniel, 1987) and studies involving targeted dominant-negative TGFβIIR (Gorska et al., 2003; Joseph et al., 1999) or overexpressed TGFβ3 (Nguyen and Pollard, 2000) demonstrate that TGFβ acts to confine mammary epithelial cell expansion by inhibiting cell proliferation and inducing apoptosis. This conclusion is underlined by findings in TGFβ1+/- transgenic mice, which have < 10% wild-type TGFβ1 levels and exhibit accelerated ductal growth and alveogenesis during puberty (Ewan et al., 2002b). Thereby, active TGFβ is confined to the luminal compartment (Fleisch et al., 2006), yet the inhibitory effect of TGFβ1 on cell proliferation extends to basal stem or progenitor cells. As a mediator for the inhibitory effect of TGFβ1, the non-canonical Wnt ligand Wnt5a was identified (Roarty and Serra, 2007), exemplifying also the interplay between the different cell fate determining pathways.

RTK signaling

The superfamily of receptor tyrosine kinases (RTK) comprises 19 subfamilies, of which the epidermal growth factor receptor (EGFR) or ErbB family, the fibroblast growth factor receptor (FGFR) and the insulin-like growth factors (IGFs) and their receptors have established roles in normal mammary gland development and mammary epithelial cell fate determination (Hynes and Watson, 2010). The ErbB family of RTKs includes EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. Ligand binding causes homo- or heterodimerization of the receptors, thereby activating their cytoplasmic kinase domains and initiating signaling (Hynes and Watson, 2010). In the mammary gland, multiple EGF-family ligands and all of the ErbB receptors are expressed during ductal morphogenesis, pregnancy-induced alveogenesis, and during lactation (Schroeder and Lee, 1998). Transgenic mice with impaired EGFR exhibit reduced ductal outgrowth (Sebastian et al., 1998), whereas pellets releasing EGF can rescue ductal development in ovariectomized mice (Coleman et al., 1988). Further studies involving knockout mice and transplantation procedures support a crucial role of stromal EGFR in mediating the effects of estrogen, downstream of estrogen-induced amphiregulin release from mammary epithelial luminal cells and upstream of FGF2 and FGF7 secretion (Sternlicht et al., 2005) (see Figure 3.2.1a).

The FGF family includes 22 ligands and four receptors, FGFR1-4. During ductal morphogenesis, multiple FGFs, including FGF2 and FGF7, as well as FGFR1 and FGFR2 are expressed (Hynes and Watson, 2010; Schwertfeger, 2009). FGFR2 signaling is important for embryonic mammary placode and bud formation, and mice deficient in the isoform FGFR2b fail to develop mammary placodes 1, 2, 3 and 5 (Mailleux et al., 2002). The requirement for FGFR signaling in mammary stem cell maintenance postnatally is supported by the

competitive disadvantage of mammary epithelial cells with deleted FGFR1 and FGFR2 in transplantation experiments (Pond et al., 2013).

Amongst the insulin-like growth factors (IGFs), IGF1 has an important role downstream of GH whereas IGF2 is expressed in response to prolactin signaling and acts to induce cyclinD1 expression (see above) (Brisken et al., 2002). Notably, IGF1 signaling in the mammary gland is fine-tuned by the secretion of IGF-binding proteins by mammary epithelial and stromal cells (Flint et al., 2008).

Apart from these main cell fate determining pathways in the mammary gland known to date, several accessory signaling pathways are believed to have integrative roles. Hedgehog signaling is one example (Sternlicht, 2006). However, the details of many interactions remain to be determined and are under current investigation by several research groups.

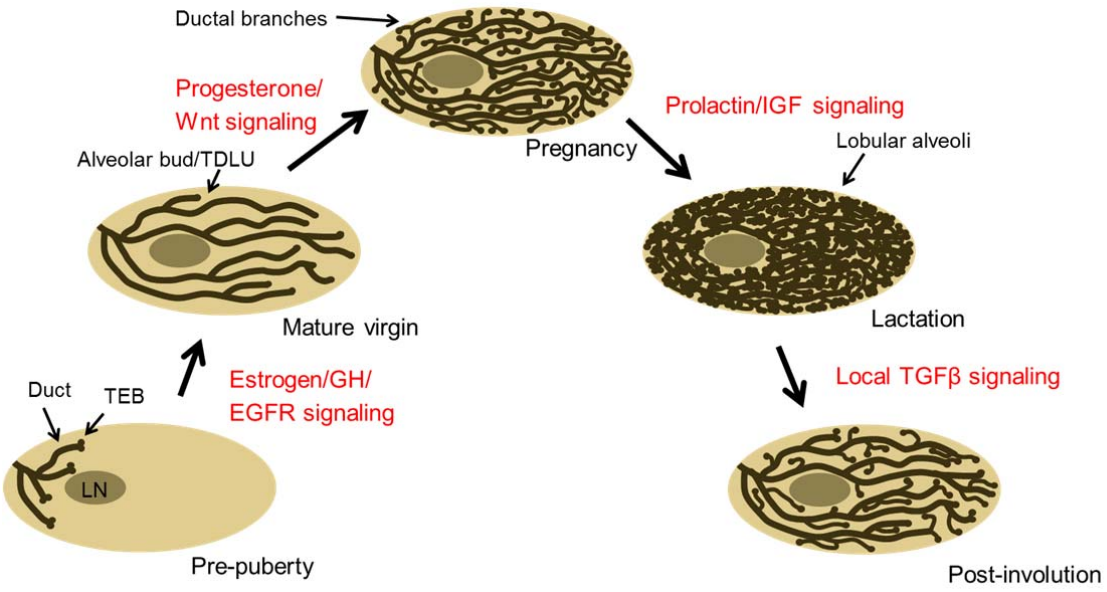

Figure 3.2.2 Overview of hormone, growth factor and cell fate determining signaling pathways in mammary gland development

Schematic illustration of mammary gland developmental stages and the corresponding critical signaling cascades.

3.3 Breast cancer

3.3.1 Pathogenesis and subtypes of breast cancer

Aberrant normal mammary gland growth and homeostasis may lead to cancer. Breast tumors arise from mammary epithelial cells (Sims et al., 2007) and are thought to progress from flat epithelial atypia (FEA), via atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) (Bombonati and Sgroi, 2011). Cause of death is usually metastasis of malignant tumor cells to distant organs such as bone, lung, liver, and brain (Nguyen et al., 2009). Clinically, breast cancers are classified according to tumor size, histological grade, node status, and expression of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2/ErbB2) (Di Cosimo and Baselga, 2010; Sotiriou and Pusztai, 2009). Apart from this conventional classification method which directs current treatment strategies, the development of large scale molecular profiling techniques has led to the identification of distinct breast cancer subtypes, which vary according to their intrinsic aggressiveness and their prognosis (Morris and Carey, 2007). Based on global gene expression analyses, the following subtypes of breast cancer are distinguished: luminal A, luminal B, HER2- or ErbB2-enriched, basal-like, normal-like and claudin-low (Herschkowitz et al., 2007; Perou and Borresen-Dale, 2011; Perou et al., 2000; Prat et al., 2010; Sorlie et al., 2001). On the basis of copy number variants and single

nucleotide polymorphisms, further breast cancer subgroups are defined, which yet remain to be characterized in detail (Curtis et al., 2012).

The most frequently diagnosed breast cancers are hormone receptor-positive tumors and fall into the luminal A or the luminal B breast cancer subtypes. They are characterized by a gene expression profile similar to luminal mammary epithelial cells (Perou et al., 2000) and are often low-grade (Perou and Borresen-Dale, 2011). Luminal A tumors comprise 40-50% of all breast cancers (Carey et al., 2006; Millikan et al., 2008; Morris et al., 2007) and exhibit high expression of ER and ER-regulated genes (including PR) with low expression of proliferation markers such as Ki-67 (Hu et al., 2006b; Sorlie et al., 2001; Sorlie et al., 2003). They are associated with responsiveness to hormonal therapy and good prognosis (Fan et al., 2006; Loi et al., 2007; Sotiriou et al., 2003). In contrast, luminal B tumors (accounting for approximately 10% of all breast cancers), exhibit lower expression of ER and ER-regulated genes (Perou and Borresen-Dale, 2011), are much more proliferative and less responsive to hormonal therapy (Sims et al., 2007; Sotiriou and Pusztai, 2009).

The HER2- or ErbB2-enriched breast cancer subtype represents 10-25% of all breast cancers and is characterized by increased HER2/ErbB2 expression (Berger et al., 1988; Carey et al., 2006; Slamon et al., 1987; van de Vijver et al., 1987). Further features of this subtype comprise low expression of basal-like and/or hormone receptor-regulated genes, high expression of proliferation markers and high incidence (> 40%) of mutations in p53, a key mediator of cellular response to DNA damage (Perou and Borresen-Dale, 2011). HER2- or ErbB2-enriched breast cancers are typically high-grade tumors, and before the era of HER2-targeted therapies, this subtype was associated with poor prognosis (Parker et al., 2009; Sorlie et al., 2001).

Basal-like tumors tend to lack HER2/ErbB2, ER, PR expression and represent the major part of what is clinically known as “triple-negative” cancers (Di Cosimo and Baselga, 2010; Perou and Borresen-Dale, 2011). They comprise 15-20% of breast cancer cases and are characterized by aggressive clinical behavior and high incidence of metastasis (Di Cosimo and Baselga, 2010). Apart from the expression of basal epithelial markers (cytokeratin 5, 6, 14, 17, c-kit, vimentin, p-cadherin etc.) which accounts for their name, basal-like tumors exhibit a strong “proliferation signature” and may be marked by a high p53 mutation rate (> 50%) and aberrant BRCA1 activity, a gene essential for proper DNA repair and chromosomal stability (Perou and Borresen-Dale, 2011; Turner et al., 2007). In line with the latter are findings of most BRCA1-associated tumors being triple negative and basal-like (Anders and Carey, 2008). Although the subtype of triple negative and basal-like breast cancers often responds well to chemotherapy, the overall prognosis is poor (Di Cosimo and Baselga, 2010; Rouzier et al., 2005; Sims et al., 2007).

Normal-like breast cancers resemble the normal breast in their gene expression. They are poorly characterized, but exhibit a better prognosis than basal-like cancers despite their failure to respond to neoadjuvant chemotherapy (Fan et al., 2006; Rakha et al., 2008; Rouzier et al., 2005; Sorlie et al., 2001).

The claudin-low subtype of breast tumors has been identified more recently (Herschkowitz et al., 2007) and is characterized by reduced expression of cell adhesion and tight junction genes and by increased expression of mesenchymal genes (Perou and Borresen-Dale, 2011). Such decreased epithelial and increased mesenchymal features are typical for normal mammary stem cells. This finding was influential for one of the current hypotheses regarding the cells of origin of specific breast cancer subtypes.

Comparing the gene expression profiles of normal FACS-sorted mammary cell subpopulations with breast cancer subtypes showed similarities between basal stem/progenitor cells and claudin-low/normal-like cancers, between luminal progenitor cells and basal-like tumors, and between differentiated luminal cells and luminal A/B cancers (Lim et al., 2009) (see Figure 3.3.1). However, whilst deductions from such correlations on potential cells of origin are attractive, direct tumorigenic capacity needs to be demonstrated in lineage tracing or clonality experiments. An alternative hypothesis for cells of cancer origin is based on specific mammary cell subpopulations showing differentiation plasticity during tumorigenesis. According to this theory, the gene expression profiles of the breast cancer subtypes do not reflect the features of their actual cells of origin (Visvader, 2009).

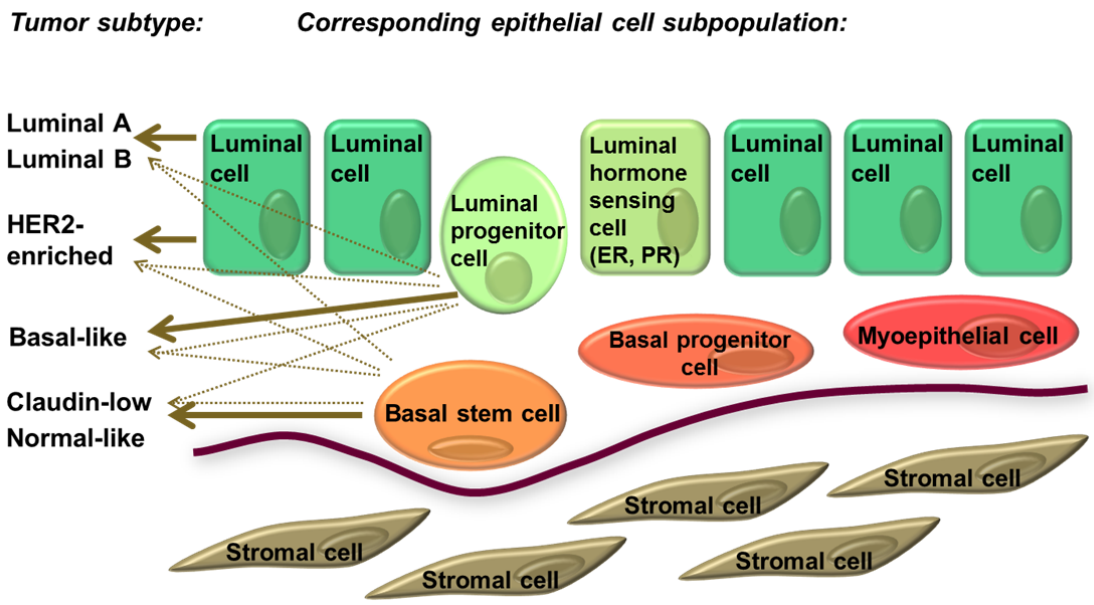

Figure 3.3.1 Breast cancer subtypes and potential cells of origin

Gene expression comparisons suggest a relationship of breast cancer subtypes and cells of origins as depicted with full arrows. In contrast, hypotheses based on mammary cell plasticity

and differentiation promote mechanistic models where tumors arise from stem or progenitor cells (dotted arrows).

3.3.2 Cell fate determining signaling pathways and breast carcinogenesis

Amongst the main cell fate determining signaling pathways identified in the mammary gland, aberrantly increased Wnt signaling plays a central role in mammary oncogenesis (Visvader, 2009). Transgenes encoding components of the Wnt signaling pathway target undifferentiated mammary progenitor cells for tumorigenesis (Li et al., 2003; Liu et al., 2004; Teuliere et al., 2005) and render them resistant to radiation therapy (Woodward et al., 2007). Moreover, the Wnt/β-catenin pathway is activated following knockdown of the tumor suppressor gene PTEN in human breast cells (Korkaya et al., 2009), and most invasive breast carcinomas exhibit downregulation of the secreted Wnt inhibitor Secreted Frizzled-Related Protein1 (Sfrp1) (Roarty and Rosen, 2010; Ugolini et al., 2001), further underscoring the connection between Wnt signaling and breast carcinogenesis.

Similar to increased Wnt signaling, elevated and inappropriate RTK signaling in the mammary gland is strongly linked to oncogenesis. ErbB2 or HER2 overexpression accounts for up to 25% of breast cancer cases (see above), transgenic mice overexpressing EGFR develop hyperplasias and mammary cancers (Brandt et al., 2000), EGFR is expressed in 30-60% of basal-like breast cancers (Reis-Filho and Tutt, 2008; Thike et al., 2010) and clinical trials with EGFR inhibitors are ongoing (ClinicalTrial.gov). Apart from the key role of EGFR signaling in breast carcinogenesis, FGF and IGF-1 signaling are also implicated in tumor initiation and progression (reviewed in (Hynes and Watson, 2010)).

Unlike Wnt and RTK signaling, which both have clear tumor-promoting effects in the mammary gland, the roles of Notch and TGFβ signaling are equivocal, and oncogenic as well as tumor-suppressing properties are observed for both pathways. Study results regarding altered Notch signaling in breast tumor tissue and its prognostic value are ambiguous (Dickson et al., 2007; Han et al., 2011; Lee et al., 2007; Li et al., 2010; Mittal et al., 2009; Zardawi et al., 2010). In line with a tumor-suppressive role of Notch signaling, high Notch2 mRNA expression is associated with good clinical outcomes (Parr et al., 2004) and ectopic expression of the active intracellular domain of Notch2 (N2ICD) has been demonstrated to reduce growth and enhance apoptosis of basal-like breast cancer cells (O'Neill et al., 2007). Interestingly, recent evidence suggests that the tumor-suppressive role of Notch signaling may in part function by counteracting WNT/β-catenin signaling (Kim et al., 2012). In support of an oncogenic role, positive associations are observed for Notch2 and HER2 expression in invasive human breast cancers (Florena et al., 2007). Moreover, overexpression of the active intracellular domain of Notch4 (N4ICD) increases cellular proliferation in the same basal-like breast cancer cells which are inhibited by N2ICD (O'Neill et al., 2007), transgenic mice expressing constitutively active N1ICD or N3ICD in mammary epithelium form mammary tumors (Hu et al., 2006a), and Notch1 inhibition results in mammary tumor regression in transgenic mouse models (Simmons et al., 2012). Together, the data suggest cell type- and paralog-specific effects of Notch signaling in carcinogenesis, but more studies are warranted to define the exact roles of distinct Notch paralogs on specific mammary epithelial cell subtypes.

Like Notch signaling, TGFβ signaling also has paradoxical roles in mammary tumorigenesis. TGFβ regulates tumor proliferation by mediating cell cycle arrest (Dunphy et al., 2011; Ewan et al., 2005). It inhibits telomerase activity (Li et al., 2007; Li and Liu, 2007) and induces

apoptosis (Bierie et al., 2009; Ewan et al., 2002a), thereby suppressing tumorigenesis. In contrast, TGFβ promotes epithelial-to-mesenchymal transition (EMT) and induces metastatic and invasive properties in tumor cells resistant to its tumor-suppressing roles (Dumont and Arteaga, 2000; Muraoka-Cook et al., 2005).

3.4 Pregnancy and risk of breast cancer

3.4.1 Epidemiological data in humans and experimental studies in rodents

An extensive body of epidemiological studies has established a strong and life-long breast cancer protective effect of early full-term pregnancy in humans (Albrektsen et al., 2005; Kelsey et al., 1993; MacMahon et al., 1970; McPherson et al., 2000). This protective effect is at least 50% with a pregnancy occurring before the age of 20 years. Interestingly, pregnancy-induced breast cancer protection is negligible with first full-term pregnancies at ages between 30 and 34 and is even reversed to an overall increased risk of developing mammary tumors with first pregnancies after the age of 35 years (MacMahon et al., 1970; Trichopoulos et al., 1983). Apart from early age at first pregnancy, multiple pregnancies and extensive breastfeeding also decrease a woman's breast cancer risk, although to a relatively small degree: the time of breastfeeding is inversely associated with breast carcinoma and every additional pregnancy confers a further 10-13% of protection against breast cancer (Lambe et al., 1996; Ursin et al., 2004). Importantly, before the protective effect of pregnancy becomes apparent, there is an initial increase in breast cancer risk immediately after parturition in women over 25 years (Albrektsen et al., 2005). This transient and pregnancy-associated elevation in breast cancer risk is most pronounced in women older than 30 years (Albrektsen et al., 2005; Lambe et al., 1994; Schedin, 2006) and may account at least in part for the

overall increase in breast cancer risk observed in women older than 35 years at first full-term pregnancy.

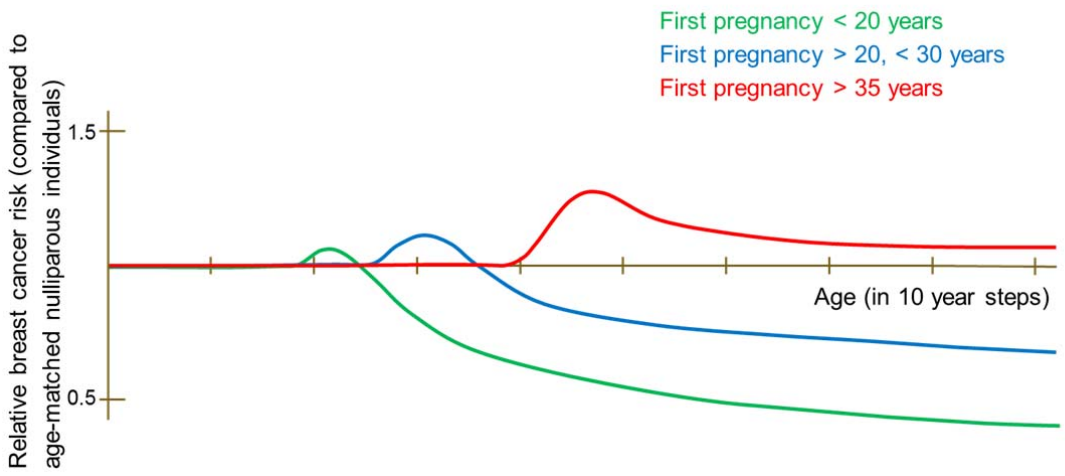

Figure 3.4.1 Effect of pregnancy and age at first birth on breast cancer risk

Schematic illustration of the epidemiologic study results from the last decades demonstrating that (1) early pregnancy decreases the breast cancer risk in the long-term, (2) the breast cancer protective effect of pregnancy is greater the earlier the pregnancy has occurred, (3) pregnancy leads to a transient increase in breast cancer risk following parturition, and (4) pregnancy-associated increase in breast cancer risk becomes more pronounced with late age at pregnancy.

Further analysis of the epidemiologic data for breast cancer subtypes shows that parity specifically protects against ER+/PR+ breast cancer. A meta-analysis on two cohort, six population-based case-control and two hospital-based case control studies affirmed that each birth reduces the risk of ER+/PR+ breast cancer by 11% and that women in the highest age at first birth category have a 27% higher life-time risk of developing ER+/PR+ tumors compared to women in the youngest age at first birth category. In contrast, neither parity

itself nor age at first birth affects the risk of ER-/PR- breast cancers (Ma et al., 2006). In line with this conclusion, a recent analysis pooling data from 34 studies and including 35,568 invasive breast cancer case patients found clear inverse associations between parity/early age at first birth and ER+ tumors (Yang et al., 2011). Regarding breastfeeding and tumor subtypes, data is less definitive, but recent results point towards a protective effect of breastfeeding mainly against luminal breast cancers (Turkoz et al., 2012). With respect to the familial breast cancers BRCA1 and BRCA2, study results are equivocal, but the majority of large studies in Western Europe demonstrate a protective effect of parity against both BRCA1 and BRCA2 associated breast cancers (Andrieu et al., 2006; Antoniou et al., 2006; Milne et al., 2010). Given that BRCA1 tumors tend to be ER- (see above and (Foulkes et al., 2004; Musolino et al., 2007), this finding may seem surprising. However, BRCA1 carriers are protected against breast cancer by oophorectomy (Eisen et al., 2005; Rebbeck et al., 1999), suggesting an indirect hormone-dependency of these tumors which may account for the protective effect of an early pregnancy.

Parity-induced protection against breast cancer is well-established not only in humans, but also in experimental rodent models. In rats or mice, high incidence of mammary cancers is observed in virgin animals after carcinogen administration, but the same carcinogens fail to induce tumors when given to rats or mice after a full-term pregnancy in greater than 75% of cases (Moon, 1969; Russo and Russo, 1980; Sinha et al., 1988; Thordarson et al., 1995; Yang et al., 1999). Moreover, hormonal mimicry of pregnancy, by treatment with estrogen and progesterone or human chorionic gonadotropin for at least 21 days, has proven equally effective as early full-term pregnancy for preventing mammary cancers in carcinogen-treated rodents (Grubbs et al., 1985; Guzman et al., 1999; Rajkumar et al., 2001; Russo et al., 1990). More recently, such hormone treatment has been shown to prevent mammary tumorigenesis

in two different genetically engineered mouse models of breast cancer (the p53-null mammary transplant model and the Her2/Neu transgenic mouse model) (Rajkumar et al., 2007).

With respect to the length of pregnancy required to confer breast cancer protection, limited studies in humans suggest that interrupted pregnancies neither raise nor reduce the risk of mammary tumorigenesis (Erlandsson et al., 2003; Goldacre et al., 2001; Henderson et al., 2008; Melbye et al., 1997; Michels et al., 2007), whereas two rodent studies gave ambiguous results: One showed that pregnancy terminated at day 5, 10, or 15 leads to an intermediate mammary cancer incidence following carcinogen administration of 48, 50, and 45% as compared to 70-88% in virgins and 14% in parous control mice (Sinha et al., 1988). The other study observed no protective effect for pregnancy interrupted at day 12 (Russo and Russo, 1980). The divergent results may be explained by differences in the size of the animal groups used and thus differences in the statistical powers, and/or by the specific time points of carcinogen treatment (21 days versus 15 days after end of hormone treatment). Further experiments are needed and may provide hints regarding the mechanism underlying the protective role of parity against breast cancer.

3.4.2 Hypothetical mechanisms of breast cancer protective effect

Although the breast cancer protective effect of an early full-term pregnancy or its hormonal mimicry has been known for decades, the cellular and molecular mechanisms underlying this phenomenon remain unclear. The prevailing hypotheses involve two cell non-autonomous and three cell autonomous mechanisms. Thereby, the individual theories are not mutually exclusive, and a combination of several processes is probably required to bring about the full protective effect.

Hypotheses based on cell non-autonomous mechanisms specify potential persistent changes in circulating hormones and/or changes in the stromal composition of the mammary gland as crucial parity-induced alterations resulting in decreased propensity for breast tumorigenesis (Medina, 2005; Schedin et al., 2004; Thordarson et al., 1995). Due to diurnal, cyclical, and age-dependent changes, studies on hormone levels require especially large cohorts in humans and/or good control measures in rodents. Whereas no clear and reproducible changes in estrogen and progesterone hormone levels are reported after pregnancy, prolactin (PRL) levels seem decreased at least transiently in parous women, a finding reproduced in some but not all rodent studies (Bernstein et al., 1985; Bridges and Byrnes, 2006; Bridges et al., 1993; Dorgan et al., 1995; Eliassen et al., 2007; Ingram et al., 1990; Kwa et al., 1981; Musey et al., 1987; Thordarson et al., 1995). Moreover, the growth hormone (GH) – insulin growth factor 1 (IGF-1) axis appears persistently suppressed after pregnancy in rats (Thordarson et al., 1995). Interestingly, diminished PRL and GH secretion induces regression of mammary tumors (Rose et al., 1983), and virgin GH-deficient rats are refractory to mammary carcinogenesis (Swanson and Unterman, 2002), whereas increased levels of PRL and GH or IGF-1 are associated with elevated incidence of mammary carcinogenesis in several studies (Harvey, 2012; Ingram et al., 1990; Thordarson et al., 2004). Such findings suggest a possible role of prolactin and GH in parity-induced breast cancer protection. Furthermore, long-lasting alterations of the mammary stroma extracellular matrix composition (ECM) and its signaling have been observed in rodent mammary glands (Schedin et al., 2004). Notably, the matrix of parous animals restricts glandular morphogenesis in 3-D *in vitro* cultures, thus suggesting the presence of growth – and possibly also tumor – suppressing factors in the ECM of parous individuals (Schedin et al., 2004).

With respect to cell autonomous processes, hypotheses for parity-induced breast cancer protection are based on potential changes in differentiation state and hormone responsiveness of the entire mammary gland or alterations in cell fates of specific mammary epithelial cell subpopulations (Britt et al., 2007; Medina, 2005; Russo and Russo, 2011). Regarding mammary gland differentiation, it was postulated that pregnancy- or pregnancy hormones-induced terminal differentiation of the mammary gland removes cancer-susceptible cells, thereby decreasing the gland's propensity to form tumors (Russo and Russo, 1997; Russo et al., 1982). This hypothesis is supported by studies examining genome-wide expression profiles of entire lobular breast tissues of women or entire mammary glands of rodents, which demonstrate a clear increase in the expression of differentiation genes in breast tissues or mammary glands from parous individuals (Blakely et al., 2006; D'Cruz et al., 2002; Russo et al., 2008). However, although attractive, mammary gland differentiation per se cannot explain all observed aspects, since neither placental lactogen nor perphenazine, both of which cause the mammary gland to differentiate, protect against mammary tumorigenesis (Guzman et al., 1999; Medina, 2005). Furthermore, pregnancy or its hormonal mimicry does not lead to persistent morphologically distinguishable differentiated cells (Sinha et al., 1988).

Regarding the responsiveness of the mammary gland to reproductive hormones, it was suggested that a decrease in the ability of the mammary gland to sense estrogen might underlie the breast cancer's protective effect of an early pregnancy (Britt et al., 2007). This theory is consistent with the roles of estrogen and progesterone in cancer: Numerous studies relate breast cancer risk to cumulative dosage of reproductive hormones, and early menarche, late menopause, and hormone replacement therapy all increase the life-time exposure to estrogen and progesterone and similarly raise the risk of breast tumorigenesis (1997; 2012; Beral, 2003; Heiss et al., 2008; Henderson et al., 1988; Ritte et al., 2012). In contrast,

decreasing hormone exposure by oophorectomy reduces mammary cancer risk (Schneider et al., 1969). Interestingly, ERα-positive estrogen sensing cells are known to increase with age and cancerous progression (Shoker et al., 1999), thereby underscoring a possible direct relationship between mammary gland hormone responsiveness and breast cancer risk.

The most hotly debated theory for the breast cancer protective effect of an early pregnancy proposes a parity-induced change in the cell fate of specific mammary epithelial cell subpopulations. According to this hypothesis, the hormonal environment of pregnancy alters the developmental fate of a subpopulation of mammary epithelial cells by inducing persistent changes in signaling pathways, growth factors and/or other regulatory molecules. These differences reduce the cell subpopulation's proliferation potential and render it relatively resistant to tumorigenesis, whilst the capacity to form complete differentiated lobular structures during a next pregnancy is maintained (Medina, 2005). In line with the cell fate theory, mammary cells from hormone-treated animals demonstrate a block in carcinogen-induced proliferation (Sivaraman et al., 1998). Furthermore, a new mammary epithelial cell population, termed parity-identified mammary epithelial cells (PI-MECs) was found to originate from differentiating cells during pregnancy (Wagner et al., 2002). These cells are pluripotent and can contribute to mammary outgrowths in transplantation experiments, thus exhibiting properties of stem and/or progenitor cells (Boulanger et al., 2005). Conceivably, a molecular switch in mammary stem cells may explain the breast cancer protective effect of early pregnancy (Russo and Russo, 2011), since the longevity and self-renewing property of stem cells make this population a prime target for transformation and tumorigenesis (Lindvall et al., 2007; Wagner and Smith, 2005). Notably, two studies in mice have addressed the issue of stem cell numbers in parous compared to virgin mammary glands by transplanting total mammary (epithelial) cells, but reported conflicting results (Britt et al., 2009; Siwko et al.,

2008). Despite all these indications, the cell fate hypothesis has until now never been addressed directly, for example by examining the individual mammary epithelial cell subpopulations in parous and virgin tissue side by side, thereby eliminating the masking effects of other cell types.

4. AIMS OF THE WORK/SCOPE OF THESIS

In this study we tested the possibility that early pregnancy induces cell autonomous processes that can potentially explain the observed breast cancer protective effect of early parity in humans and rodents. Notably, we investigated whether early pregnancy changes the molecular properties ("cell fates") of specific mammary epithelial cell subtypes, and whether such changes can be explained by alterations of the hormone responsiveness of the mammary gland. Specifically, the following questions were addressed:

1) Does early parity induce specific changes in gene expression and signaling pathways in one or several mammary epithelial cell subpopulations?

2) Does early parity affect the *in vitro* proliferation/differentiation potential of distinct mammary epithelial cell subtypes and the *in vivo* reconstitution capacity of isolated basal mammary stem/progenitor cells?

3) Can changes in hormone (e.g. estrogen, progesterone) responsiveness of the mammary gland explain potentially altered cell fates after early pregnancy?

The studies were performed in a previously validated mouse model, which allowed precise control for reproductive factors and their timing and provided a genetically homogenous background.

5. RESULTS

Research article

Parity induces differentiation and reduces Wnt/Notch signaling ratio and proliferation potential of basal stem/progenitor cells isolated from mouse mammary epithelium

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5.1 Abstract

Introduction: Early pregnancy has a strong protective effect against breast cancer in humans and rodents, but the underlying mechanism is unknown. Because breast cancers are thought to arise from specific cell subpopulations of mammary epithelia, we studied the effect of parity on the transcriptome and the differentiation/proliferation potential of specific luminal and basal mammary cells in mice.

Methods: Mammary epithelial cell subpopulations (luminal Sca1$^-$, luminal Sca1$^+$, basal stem/progenitor, and basal myoepithelial cells) were isolated by flow cytometry from parous and age-matched virgin mice and examined by using a combination of unbiased genomics, bioinformatics, *in vitro* colony formation, and *in vivo* limiting dilution transplantation assays. Specific findings were further investigated with immunohistochemistry in entire glands of parous and age-matched virgin mice.

Results: Transcriptome analysis revealed an upregulation of differentiation genes and a marked decrease in the Wnt/Notch signaling ratio in basal stem/progenitor cells of parous mice. Separate bioinformatics analyses showed reduced activity for the canonical Wnt transcription factor LEF1/TCF7 and increased activity for the Wnt repressor TCF3. This finding was specific for basal stem/progenitor cells and was associated with downregulation of potentially carcinogenic pathways and a reduction in the proliferation potential of this cell subpopulation *in vitro* and *in vivo*. As a possible mechanism for decreased Wnt signaling in basal stem/progenitor cells, we found a more than threefold reduction in the expression of the secreted Wnt ligand *Wnt4* in total mammary cells from parous mice, which corresponded to a similar decrease in the proportion of Wnt4-secreting and estrogen/progesterone receptor-positive cells. Because recombinant Wnt4 rescued the proliferation defect of basal stem/progenitor cells *in vitro*, reduced Wnt4 secretion appears to be causally related to parity-induced alterations of basal stem/progenitor cell properties in mice.

Conclusions: By revealing that parity induces differentiation and downregulates the Wnt/Notch signaling ratio and the *in vitro* and *in vivo* proliferation potential of basal stem/progenitor cells in mice, our study sheds light on the long-term consequences of an early pregnancy. Furthermore, it opens the door to future studies assessing whether inhibitors of the Wnt pathway may be used to mimic the parity-induced protective effect against breast cancer.

5.2 Introduction

Pregnancy is the most significant modifiable factor known for breast cancer risk in women. Although an initial increase in risk occurs immediately after parturition in women older than 25 years, the overall lifetime risk of breast cancer decreases after pregnancy [1,2]. This protective effect is >50% if a full-term pregnancy has occurred before the age of 20 years [1]. Similarly, pregnancy and pregnancy-mimicking hormones have a strong protective effect against mammary tumors in rodents. This is true both for carcinogen-induced mammary tumors [3] and for genetically engineered mouse models of breast cancer [4].

The cellular and molecular mechanisms underlying the breast cancer-protective effect of early pregnancy remain unclear. Frequently raised hypotheses involve cell non-autonomous mechanisms such as systemic changes in circulating hormones and/or changes in the stromal composition of the mammary gland [5,6], and cell autonomous processes such as changes in the differentiation state of mammary epithelial cells [7]. Furthermore, numerous parity-induced changes in gene expression have been identified in genome-wide expression profiles of entire lobular breast tissues of women or entire mammary glands of rats and mice [8-10]. However, it is not known to what extent these tissue studies reflect alterations in gene-expression profiles of distinct mammary epithelial cell subpopulations. Hence, given that breast cancers arise from specific subpopulations of mammary epithelial cells [11], investigations of early parity-induced gene-expression changes in distinct mammary epithelial cell subpopulations are warranted.

The mammary epithelium is hierarchically organized into differentiated luminal and basal (myoepithelial) cells, luminal and basal progenitor cells, and mammary stem cells [12,13]. Whereas the latter were originally thought to lie exclusively in the basal compartment and to

be multipotent (able to form both luminal and basal epithelial cells), recent lineage-tracing experiments indicated the existence of unipotent basal and luminal mammary stem cells and identified multipotent mammary stem cells solely in the embryonic and possibly in the pregnant gland [14,15]. Distinct mammary epithelial cell subpopulations, including luminal progenitor and basal stem/progenitor cells can be isolated with fluorescence-activated cell sorting (FACS) by using specific cell-surface markers from both parous and virgin mice [16-21]. Whereas progenitor cells in general can be characterized *in vitro* by their colony-forming potential [16,22,23], the basal stem/progenitor cell subpopulation has the additional capacity to repopulate deepithelialized mouse mammary fat pads *in vivo* [16-18]. Although previous studies in total mammary epithelial cells indicated either no change or a decrease in the mammary repopulating capacity after parity [20,21], the consequences of parity on the transcriptome and functionality of specific mammary epithelial cell subpopulations have not been investigated.

Therefore, we examined in this study whether pregnancy alters the gene-expression profiles ("gene signature") and the differentiation/proliferation potentials of the various mammary epithelial cell subpopulations. The results indicate that early parity decreases *Wnt4* expression in luminal epithelial cells, leading to a reduction in the Wnt/Notch signaling ratio specifically in basal stem/progenitor cells. As expected, the decrease in the Wnt/Notch signaling ratio is associated with a concomitant strong prodifferentiation and antiproliferation phenotype in basal stem/progenitor cells. Because a decrease in Wnt signaling is known to have an anticarcinogenic effect [24,25], the findings support the hypothesis that a reduction in the Wnt/Notch signaling ratio in basal mammary stem/progenitor cells plays a role in the mitigating effect of early pregnancy on breast tumorigenesis.

5.3 Methods

5.3.1 Animals and animal experimentation

All experiments were conducted in genetically homogenous FVB/NHanHsd mice purchased from Harlan Laboratories. The mice were bred and maintained in the animal facility of the Friedrich Miescher Institute, according to the Swiss guidelines on animal experimentation. All experiments were performed under permit 2159-2, in accordance with the animal-welfare ordinance and approved by the cantonal veterinary office of Basel Stadt, Switzerland. For the early-pregnancy protocol, mice were time-mated when 42 days old and allowed to lactate for 21 days. The postweaning period until cell harvest was 40 days, unless stated otherwise. To control for the estrus cycle in the transcriptome analyses, at least five mice were grouped for gland harvesting. For immunohistochemical analyses and determination of blood progesterone levels, mice in estrus were used, as assessed by the presence of a vaginal plug after an overnight mating. Age-matched virgin control mice were maintained under the same conditions as parous mice.

5.3.2 Whole mounts

Whole mounts were prepared by fixing the glands on glass slides with methacarnoy solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 4 hours at room temperature. The mounts were hydrated by sequential incubation in ethanol solutions of decreasing concentration: 100%, (overnight), 70%, 50%, and 30% (15 minutes each), distilled water (2 × 5 minutes), and stained overnight with an aqueous solution of 2% carmine (Sigma, Buchs, Switzerland) and 5% aluminum potassium sulfate (Sigma, Buchs, Switzerland). The mounts were dehydrated in ethanol solutions (70%, 90%, 95%, and 2 × 100%, for 15 minutes each) and cleared with xylene overnight. Images were captured with an Epson Expression 1600 Pro scanner.

5.3.3 Mammary cell preparation

The fourth mammary glands from virgin and parous mice were collected after lymph node removal and pooled. Mammary epithelial organoids were prepared as described [26]. Adipocytes were removed by repeated centrifugations (300 *g*). Red blood cells (RBCs) were eliminated by incubation with 8.3 g/L ammonium chloride (pH 7.5) for 5 minutes. The number of fibroblasts in gland extracts was reduced by their attachment to polystyrene cell-culture flasks (Corning, Buchs, Switzerland) during a 45-minute incubation step in Dulbecco modified Eagle medium (Invitrogen, Zug, Switzerland) with 10% FCS (Sigma, Buchs, Switzerland) at 37°C/5% CO_2 [26]. The epithelial organoids were directly processed to single-cell suspensions by digestion in Hyclone HyQTase (ThermoScientific, Lausanne, Switzerland) with gentle pipetting for 15 minutes at 37°C. The cell suspension was filtered through a 40-µm cell strainer (BD Falcon, Basel, Switzerland), and the final cell suspension pelleted at 650 *g* for 4 minutes.

5.3.4 Cell labeling and flow cytometry

Cells were labeled as previously described [26] by using the antibodies PE-Cy7-CD45 (1:33), FITC-CD24 (1:40), PE-CD49f (1:40), and APC-Sca1 (1:40). Detailed antibody information is given later. DAPI (0.2%, Invitrogen, Zug, Switzerland) was added 10 minutes before cell sorting (1:250). FACS was carried out on a MoFlo cell sorter (Becton Dickinson, Basel, Switzerland). Cells were gated based on their forward- and side-scatter profiles (FS Area and SS Area). A time-of-flight approach (pulse width) was used to exclude doublets and higher-order cell clumps. Dead cells (DAPI bright) and immune cells ($CD45^+$) were gated out (see Additional file 1). The gate for basal stem/progenitor cells was set at the top 5% of CD49f-expressing cells, as described [19,21] (see Figure 1). Routine examination of the sorted mammary epithelial cell subpopulations showed a degree of purification higher than 95%.

5.3.5 *In vitro* colony formation assay and quantification

Freshly sorted cells of each subpopulation (500 cells) were plated onto irradiated 3T3L1 feeder cells (5,000 cells) in 24-well Primaria plates (Becton Dickinson, Basel, Switzerland). The cells were cultured in DMEM/Ham F12 (Invitrogen, Zug, Switzerland) with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Zug, Switzerland), 5 µg/ml bovine pancreatic insulin (Sigma, cell culture-tested solution, Buchs, Switzerland), 10 ng/ml mouse EGF (BD Biosciences, Basel, Switzerland), and 10 ng/ml cholera toxin (Sigma, Buchs, Switzerland). After 24 hours, the medium was renewed, and 4 days later, the colonies were fixed with acetone/methanol (1:1), washed, and rehydrated with PBS and 0.05% NaN_3. In separate rescue experiments with selected mammary epithelial cells from parous mice, the incubation medium was supplemented with recombinant mouse Wnt4 (R&D Systems, Abingdon, UK) at 500 ng/ml. For quantification, colonies were immunofluorescently stained with Krt18 and Krt14 antibodies and with Hoechst 33342. The stained colonies were imaged with a MacroFluo Z16 microscope (Leica, Heerbrugg, Switzerland) at 2× magnification, and a Z1 microscope (Zeiss, Feldbach, Switzerland) at 5× magnification. The feeder cells served as negative controls for Krt18 and Krt14 staining. The number of colonies (that is, clusters of more than three cells [23]) per well was determined manually. Colonies defined as Krt18/Krt14 double-positive were double positive over a minimum of 20% of colony area.

5.3.6 Mammary fat pad transplantation

Freshly sorted basal stem/progenitor cells were resuspended in PBS with 50% Matrigel (BD Biosciences, Basel, Switzerland) and injected (30 µl) in limiting dilution numbers into the cleared fourth mammary glands of 3-week-old syngeneic female mice. Glands from recipient mice were harvested 8 to 9 weeks after transplantation, processed as whole mounts (see earlier), and scanned with an Epson Expression 1600 Pro scanner. The number of outgrowths

was counted, with an outgrowth defined as an epithelial structure with ducts starting from a central point and with lobules and/or terminal end buds (see Additional file 2) [27].

5.3.7 Microarray analyses

Microarray analyses were performed on unsorted total mammary cell suspensions (see Figure 2) and on FACS-sorted mammary cell subpopulations (see Figures 3 and 4, as well as Table 1). For unsorted mammary cells, RNA was extracted by using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was removed by using gDNA Eliminator Mini Spin Columns (Qiagen, Hilden, Germany). The RNA concentration was measured with a Nanodrop 1000 machine, and RNA quality determined with an Agilent 2100 bioanalyzer and RNA Nano Chips. Aliquots of 100 ng of isolated total RNA were amplified by using the Ambion WT Expression kit (Ambion, Zug, Switzerland). For FACS-sorted mammary epithelial cell subpopulations, total RNA was isolated by using the Arcturus PicoPure Frozen RNA Isolation Kit (Life Technologies, Zug, Switzerland). Genomic DNA was removed by using RNase-Free DNase (Qiagen, Hilden, Germany), the RNA concentration determined by using the RiboGreen Assay, and RNA quality assessed by using an Agilent 2100 bioanalyzer and RNA Pico Chips. Total RNA was used as the input for synthesis of amplified cDNA with the NuGen Ovation Pico WTA System (NuGen Inc., Leek, The Netherlands).

Resulting double-stranded cDNA was fragmented and labeled by using the Affymetrix GeneChip WT Terminal Labeling kit (Affymetrix, High Wycombe, UK). Affymetrix Gene Chip Mouse gene 1.0 ST microarrays were hybridized according to the GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, High Wycombe, UK) with a hybridization time of 16 hours. The Affymetrix Fluidics protocol FS450_0007 was used for washing. Scanning was performed with Affymetrix GCC Scan Control Software v.

3.0.0.1214 on a GeneChip Scanner 3000 7G with autoloader. Arrays were normalized, and probeset-level expression values calculated with R/Bioconductor's (v2.14) “affy” package by using the rma() function. Differential gene expression between experimental and control samples was determined by using linear modeling as implemented in the R/Bioconductor package “limma.” For general analysis of gene expression in total mammary cell suspensions and in FACS-sorted mammary cell subpopulations, we used the cut-off linear fold change >1.5, adjusted *P* value <0.05, and average linear expression between conditions greater than 4. To determine the 10 most up- or downregulated genes in FACS-sorted mammary epithelial cell subpopulations, a cut-off of linear fold change >2.0, adjusted *P* value <0.001, and average linear expression between conditions ave>4 was used. For FACS-sorted mammary epithelial cell subpopulations both resulting lists of differential genes were imported into Ingenuity IPA (Ingenuity, content version 12710793) for pathway analysis. Gene-set enrichments in FACS-sorted mammary epithelial cell subpopulations were determined by using the JAVA application from the Broad Institute v2.0 [28,29] and gene sets v2.5 and v3.0, as well as custom gene sets (see Additional file 3).

The microarray data from this publication have been submitted to the NCBI Gene Expression Omnibus [30] and are deposited as GSE40875 (mouse mammary cell subtypes), GSE40876 (total mammary epithelial cells in mice), and GSE40877 (both total mammary epithelial cells and mammary cell subtypes in mice).

5.3.8 Quantitative PCR

RNA was isolated as described earlier and converted to cDNA by using the WT-Ovation Exon Module Version 1.0 (NuGen Inc., Leek, The Netherlands). Real-time PCR was performed on the unamplified cDNA corresponding to the specified number of cells or on 25

ng of amplified cDNA (by using the NuGen Ovation Pico WTA System (NuGen Inc., Leek, The Netherlands) for amplification). The TaqMan probe-based system was applied in combination with the TaqMan Universal PCR Master Mix (Applied Biosystems, Zug, Switzerland). The probe IDs are given later. Cycling was performed with 7500 Fast and Step OnePlus Real-Time PCR Systems (both from Applied Biosystems, Zug, Switzerland).

5.3.9 Probe IDs for quantitative PCR

These were the probes: Areg (Mm00437583_m1), Axin2 (Mm00443610_m1), B2M (Mm00437762_m1), Cdk8 (Mm01223097_m1), Cel (Mm00486975_m1), Csn1s1 (Mm00514430_m1), Csn2 (Mm04207885_m1), Dsc2 (Mm00516355_m1), Dusp1 (Mm00457274_g1), Esr1 (Mm00433149_m1), Fst (Mm00514982_m1), Gata3 (Mm00484683_m1), Hprt (Mm00446968_m1), Id3 (Mm00492575_m1), Igfbp3 (Mm_01187817_m1), Jag2 (Mm01325629_m1), Krt8 (Mm00835759_m1), Krt14 (Mm00516876_m1), Krt15 (Mm00492972_m1), Krt19 (Mm00492980_m1), Lgr5 (Mm00438890_m1), Ltf (Mm00434787_m1), Maml2 (Mm00620617_m1), Myc (Mm00487804_m1), Pgr (Mm00435628_m1), Sfrp1 (Mm00489161_m1), Ubc (Mm01201237_m1), Vcan (Mm01283063_m1), Wnt4 (Mm01194003_m1).

5.3.10 Immunofluorescent staining

For single-cell staining, freshly sorted cells were allowed to air-dry on poly-L-lysine-coated slides and stored at -20°C. The dried cells were blocked with PBS, 2.5% goat serum, and 0.05% NaN_3 for 60 minutes under UV light. The UV treatment was used to attenuate residual fluorescence from bound FACS antibodies. Primary antibody staining was performed overnight at 4°C by using Krt18 antibody (1:1,000) and Krt14 antibody (1:500) as luminal and basal cell markers, respectively. Secondary antibody staining was carried out for 60

minutes at room temperature by using anti-guinea pig Ig-Alexa488 (1:500) and anti-rabbit Ig-Alexa546 (1:1,000). Hoechst 33342 (0.5 µg/ml; Invitrogen, Zug, Switzerland) staining was performed for 10 minutes at room temperature. The stained cells were mounted with ProLong Gold antifade reagent (Invitrogen, Zug, Switzerland) and imaged with a Z1 microscope (Zeiss, Feldbach, Switzerland) at 63× magnification. No primary antibody was added as a negative control.

5.3.11 Immunohistochemistry

The fourth mammary glands of parous or age-matched virgin control mice were collected 40 days after weaning from mice in estrus. The glands were fixed in 4% PFA and embedded in paraffin. For immunostaining with ERα and PR, the sections were dewaxed and subjected to antigen retrieval by boiling in 10 m*M* citrate buffer for 10 minutes. Subsequently, the sections were cooled to room temperature, quenched for 10 minutes with PBS and 3% H_2O_2, and blocked for 30 to 60 minutes with PBS and 2.5% NGS. ERα and PR primary antibody staining was performed overnight at 4°C at a 1:1,000 (ERα) and a 1:200 (PR) dilution. Secondary antibody staining was carried out for 30 to 60 minutes at room temperature with biotinylated anti-rabbit IgG. Immunohistochemistry for versican, β-catenin, and p21 was performed on the Ventana DiscoveryXT instrument (Roche Diagnostics, Rotkreuz, Switzerland) by using the Research IHC Dap Map XT procedure. In brief, dewaxing was performed in the machine, and slides were pretreated with mildCC1 (versican and p21) or standardCC1 (β-catenin) (Roche Diagnostics, Rotkreuz, Switzerland). Primary antibodies were incubated at 37°C for 1 hour at the following dilutions: versican, 1:50; p21, 1:50; and β-catenin, 1:500. As secondary antibody, Immpress, an anti-rabbit HRP conjugated polymer, was used. All slides were counterstained with Hematoxylin II (Roche Diagnostics, Rotkreuz, Switzerland) and Bluing Reagent (Roche Diagnostics, Rotkreuz, Switzerland). Images were

captured at 20-fold (ERα or PR) or 40-fold (versican, β-catenin, p21) magnification by using an Eclipse E600 microscope (Nikon, Egg, Switzerland). For quantification of ERα and PR positivity, at least 2,000 epithelial cells per mouse were counted. For quantification of nuclear β-catenin and p21, at least 500 epithelial cells per mouse were counted. The quantification of versican was performed with the MATLAB software by using color segmentation based on Mahalanobis distance to determine the pixels with a particular RGB-color distribution [31].

5.3.12 Determination of blood progesterone concentrations

Blood was collected from the right atrium of mice in estrus in EDTA-covered tubes (Sarstedt, Numbrecht-Rommelsdorf, Germany), and plasma was extracted by centrifugation at 1,500 *g* for 15 minutes at 4°C. Progesterone concentrations were assessed with ELISA, as specified by the manufacturer's guidelines (DRG, catalog no. EIA-1561, Marburg, Germany).

5.3.13 Antibodies

For flow cytometry, the following anti-mouse antibodies were used: PE-Cy7-CD45 (clone 30-F11), FITC-CD24 (clone M1/69), PE-CD49f (clone GoH3) (all from BD Pharmingen, Basel, Switzerland), and APC-Sca1 (clone E13-161.7, from Biolegend, San Diego, USA).

For immunofluorescent staining, the primary anti-mouse antibodies used were guinea pig keratin 18 (Krt18; Fitzgerald; catalog no. GP11, North Acton, USA) and rabbit keratin 14 (Krt14; ThermoScientific, catalog no. RB-9020, Lausanne, Switzerland). The secondary antibodies used were anti-guinea pig Ig-Alexa488 and anti-rabbit Ig-Alexa546 (Molecular Probe, Invitrogen, Zug, Switzerland).

For immunohistochemistry staining, the primary antibodies included estrogen receptor alpha (ERα; Santa Cruz Biotechnology; catalog no. SC-542, Dallas, USA), progesterone receptor (PR; Clone SP2, ThermoScientific; catalog no. RM-9102, Lausanne, Switzerland), versican (Millipore/Chemicon; catalog no. ab1033, Billerica, USA), β-catenin (Cell Signaling; catalog no. 9587, Danvers, USA), and p21 (Abcam; catalog no. ab2961, Cambridge, UK). The secondary antibodies used were biotinylated anti-rabbit IgG (Vector Labs; catalog no. BA-1000, Petersborough, UK) and anti-rabbit HRP conjugated polymer Immpress (Vector Labs; catalog no. MP-7401, Petersborough, UK).

5.3.14 Statistics

The limited dilution transplantation data were analyzed statistically as published previously [18]. The two-tailed unpaired Student *t* test was used to determine statistical significance of comparisons.

5.3.15 Motif Activity Response Analysis (MARA)

The MARA model [32] combines knowledge of gene-expression levels (measured by microarray) with transcription-factor binding sites to answer the question of which transcription factors are driving expression changes in mammary stem/progenitor cells in parous as compared with age-matched virgin control mice. Specifically, log-expression levels of all genes present on the microarray were modeled as linear combinations of transcription factor activities. The coefficients of these combinations were determined by the number of transcription-factor binding sites in the proximal promoter regions. For each transcription-factor binding motif *m* and each sample (microarray) *s*, we estimated the activity A_{ms} with the corresponding error. Furthermore, we quantified the significance of activity change of each binding motif in parous as compared with virgin control mice.

5.4 Results

5.4.1 Early pregnancy decreases luminal Sca1^{+} cells, but does not change the proportions of the other mammary epithelial cell subpopulations

To investigate the influence of early parity on the proportions of mammary epithelial cell subtypes, we first established FACS profiles of epithelial cell subpopulations in virgin FVB control mice [21,26]. Luminal CD24^{+High} Sca1^{+} cells, luminal CD24^{+High} Sca1^{-} cells, basal CD24^{+Low} Sca1^{-} CD49f^{High} cells, and basal CD24^{+Low} Sca1^{-} CD49f^{Low} myoepithelial cells were isolated from these mice (Figure 1A). Use of the established cell markers keratin 18 (Krt18) and keratin 14 (Krt14) confirmed the luminal and basal origin of the isolated cell subpopulations, and qPCR for *CD49f* and *Sca1* affirmed the purity of the isolated cell subpopulations (see Additional file 4). Because basal CD49f^{High} cells are considered to be enriched for basal mammary stem/progenitor cells in virgin mice [19,21], the term "basal CD49f^{High} cells" is used synonymously with "basal stem/progenitor cells" throughout this article. To extend these analyses to parous mice, we confirmed that involution was complete 28 and 40 days after weaning (see Additional file 5). To allow a margin of safety, 40 days after weaning was used for all subsequent cell-isolation experiments in a standardized parturition protocol with mating at 42 days (Figure 1B). The FACS profiles of epithelial cell subpopulations from parous mice and age-matched virgin control mice were similar (Figure 1C, D; Additional file 1) with the exception of luminal Sca1^{+} cells, which decreased by about 50% in parous mice ($P = 0.02$). Of note, luminal Sca1^{+} cells have been shown to be enriched for hormone receptor-positive cells [17]. This was verified by qPCR (see Additional file 6). These data demonstrate that the adopted experimental procedure permits the isolation of all epithelial cell subpopulations from parous mice, including basal stem/progenitor cells, at levels adequate for transcriptomic and functional analyses.

5.4.2 Parity upregulates differentiation genes in all cell subpopulations and decreases the Wnt/Notch signaling ratio in the basal stem/progenitor cell subpopulation

Next we investigated the effects of early pregnancy on the gene-expression profiles of the isolated mammary epithelial cell subpopulations. To control for the effect of the cell-isolation procedure on gene expression, we performed first a transcriptome and cluster analysis in non-FACS-sorted total mammary cell suspensions from age-matched virgin and parous mice (Figure 2A). The analysis showed that pregnancy induces an upregulation of many immunoglobulin and differentiation genes (for example, whey acidic protein *Wap*, and carboxyl ester lipase *Cel*) and a downregulation of growth factors (for example, amphiregulin *Areg*, betacellulin *Btc*, tumor-associated calcium signal transducer 2 *Tacstd2*), and extracellular matrix (ECM) elements (for example, laminin, gamma 2 *Lamc2*, desmocollin 2 *Dsc2*). These data are consistent with the published pregnancy-induced gene signature determined in snap-frozen rodent mammary glands [9,10]. Thus, the gene signature was not lost during the isolation procedure, confirming the validity of our experimental system. Furthermore and most interestingly, a novel 3.4-fold downregulation of the Wnt signaling protein Wnt4 was observed in cells from parous mice (Figure 2). These microarray data were validated by qPCR for four genes, including *Wnt4*. Importantly, the expression of the luminal marker *Krt8* was not altered on parity, demonstrating that the observed changes in gene expression were independent of unspecific alterations in total luminal cell numbers (Figure 2B).

Because the number of isolated basal stem/progenitor cells for gene-profiling analysis was limited compared with the other cell subpopulations (that is, myoepithelial, luminal $Sca1^-$, and luminal $Sca1^+$ cells), it was important to evaluate the influence of different cell numbers on transcriptome analysis. As shown in Additional Material, we found that it is valid to use

cell numbers in the range of 2,000 to 50,000 cells for comparison of transcriptomes from different mammary cell subpopulations (Additional file 6). Furthermore, qPCR analysis of nonamplified and amplified cDNA for known basal (*Krt14*) and luminal (*Krt8*; *Krt19*) cell marker genes confirmed that the amplification process of the microarray analysis was unbiased (Additional file 6).

In the subsequent transcriptome analysis of FACS-sorted mammary epithelial cell subpopulations from age-matched virgin control and parous mice, all mammary epithelial cell subpopulations, except immune cell-depleted stromal cells, showed parity-induced changes in gene expression with by far the most prominent effects being scored for basal stem/progenitor cells (Figure 3A). Furthermore, although differentiation genes were upregulated in all epithelial cell subpopulations, the strongest prodifferentiation effects were seen in basal stem/progenitor cells from parous mice. For example, casein alpha 1 (*Csn1s1*), casein beta (*Csn2*), and lactotransferrin (*Ltf*) were upregulated 41-, 19-, and 14-fold, respectively (Figure 3B). Apart from these differentiation genes, the Notch co-activator *Maml2* was found among the 10 most-upregulated genes (by fold change and/or by *P* value) in basal stem/progenitor cells from parous mice. In contrast, the 10 most-downregulated genes included the Wnt target and co-receptor *Lgr5*, the Wnt target and epithelial stem cell marker keratin 15 (*Krt15*) [33], and the Wnt targets versican (*Vcan*) and *Igfbp3* [34] (Figure 3B). Extension of the analysis to all data for signaling-pathway genes revealed further Wnt target genes, which were downregulated (for example, *CD44* and follistatin (*Fst*) [35,36]), and the Wnt inhibitor *Sfrp1*, which was upregulated in basal stem/progenitor cells from parous mice. Moreover, in the same epithelial cell subpopulation of parous mice, the Notch ligand *Jag2* and the Notch target genes *Gata3*, *Id3*, and *Dusp1* were upregulated, whereas the Notch inhibitor *Itch* and the Maml2 RBP-J complex inhibitors *Cdk8* and *Pak1* [37] were

downregulated (Figure 3B). These gene-expression profiling data were validated with qPCR for several selected genes (Figure 3C). Furthermore, one of the classic Wnt target genes (versican) was examined on the protein level and found to be strongly downregulated in the basal compartment of mammary glands from parous mice (Figure 3D). Thus, the data demonstrate strong upregulation of differentiation genes, a downregulation of Wnt target genes, and an increase in Notch signaling in the basal stem/progenitor cell subpopulation of parous mice.

Further verification of these conclusions came from Motif Activity Response Analysis (MARA) of transcription factor activities (see Methods) [38]. The canonical Wnt transcription factor LEF1/TCF7 was shown to have significantly decreased activity in basal stem/progenitor cells from parous mice (Figure 4A), which is consistent with the observed decrease in canonical Wnt signaling in the previous gene-expression profile analysis.

Final confirmation of parity-induced downregulation of Wnt signaling in mammary glands was provided by immunohistochemical staining of mammary gland sections from age-matched virgin control and parous mice for β-catenin. As illustrated in Figure 4B, nuclear β-catenin was observed in basal but not in luminal mammary epithelial cells of virgin mice. In parous mice, the proportion of basal mammary epithelial cells positive for nuclear β-catenin was significantly decreased (Figure 4C). Because nuclear β-catenin is necessary for Wnt target gene expression, this finding represents an additional verification of parity-induced downregulation of Wnt signaling (Figure 3).

5.4.3 The parity-induced decrease in Wnt/Notch signaling ratio is specific for the basal stem/progenitor cell subpopulation

To assess whether the observed decrease in canonical Wnt and increase in Notch signaling were specific for basal stem/progenitor cells, we next investigated the enrichment of Wnt/Notch signaling genes over all genes altered in the various FACS-sorted mammary epithelial cell subpopulations from parous as compared with virgin control mice. Such analysis showed that canonical Wnt signaling was significantly downregulated in basal stem/progenitor but not in luminal $Sca1^-$ or luminal $Sca1^+$ cells from parous mice (Table 1; see Additional file 7). In myoepithelial cells, a trend toward a decrease in Wnt signaling was not significant when applying a very stringent cut-off (see Table 1 legend) and was probably due to contamination of the myoepithelial cell subpopulation with basal stem and/or progenitor cells. Similarly, Notch signaling was found to be significantly upregulated in basal stem/progenitor cells but in no other mammary epithelial cell subpopulation (Table 1). Whereas downregulation of Wnt and upregulation of Notch signaling were specific for basal stem/progenitor cells, similar enrichment analyses for genes involved in other signaling pathways revealed that the previously reported upregulation of the p53-p21 pathway [39] occurred in all epithelial cell subpopulations tested (Additional file 7).

Further bioinformatics analysis of the data with different software [40] provided a second line of verification of the specific decrease in Wnt signaling in basal stem/progenitor cells after parity. Performing transcription-factor activity analyses based on target gene expression, we found TCF3, an inhibitor of the canonical Wnt signaling pathway [41], to be the transcription factor with the highest z-score ($Z = 3.521$) and the protooncogene MYC to have a very low z-score ($Z = -4.108$) in basal stem/progenitor cells but not in other mammary epithelial cell subpopulations of parous mice (Figure 4D, z-score defined as $(x-\mu)/sd$ and used as a measure

for transcription factor activity based on the expression levels of target genes). These findings confirm the results obtained in the MARA analysis. Furthermore, inhibition of MYC leads to upregulation of *Sfrp1*, which in turn inhibits canonical Wnt signaling [42]. Upregulation of *Sfrp1* was observed in the microarray analysis and validated with qPCR (Figure 3B, C), thus directly reflecting the expected effects of the bioinformatic predictions.

Downregulation of canonical Wnt signaling and MYC activity would be expected to decrease the propensity for cancer, and indeed, in an analysis of biofunctions, a marked and consistent decrease in cancer-associated functions, was observed for basal stem/progenitor cells (Figure 4E), but no other mammary epithelial cell subpopulation. This potential anticancer phenotype of basal stem/progenitor cells was underscored by gene-enrichment analyses on all available pathway gene sets (see Additional file 8), which showed a strong downregulation of proliferation- and tumorigenesis-associated gene sets.

5.4.4 Parity decreases the *in vitro* clonogenic potential to the greatest extent in the basal stem/progenitor cell subpopulation

Because decreased Wnt signaling and increased Notch signaling have been shown to decrease *in vitro* and *in vivo* proliferation of basal stem/progenitor cells [43,44], we next assessed the *in vitro* colony-formation capacities of mammary epithelial cell subpopulations from parous and age-matched virgin mice (Figure 5A). In virgin control mice, luminal $Sca1^{-}$ cells had the highest colony-formation capacity, with an average of 107 colonies per well (Figure 5B). This strong clonogenic potential suggests a pronounced progenitor identity of luminal $Sca1^{-}$ cells and is consistent with previous observations in younger virgin mice [16,17]. A high colony-formation capacity was also observed for the basal stem/progenitor cells of virgin mice (63 colonies per well) (Figure 5B), which is consistent with the notion that $CD49f^{High}$

cells contain a high proportion of basal progenitor cells as well as putative mammary stem cells [45]. With the exception of luminal Sca1$^-$ cells, the colony-formation capacities of all epithelial cell subpopulations were lower in parous mice than in age-matched virgins (Figure 5B). Thereby, by far the most pronounced difference was observed in basal stem/progenitor cells (Figure 5B). A substantial decrease in the colony-formation capacity was also seen for the myoepithelial cell subpopulation, which also contains basal progenitor cells. Of note, basal stem/progenitor cells from parous mice did not die but remained as quiescent single cells or divided only once during 5 days of culture (Figure 5C).

The reduced progenitor potential of basal mammary epithelial cell subtypes and especially of basal stem/progenitor cells from parous mice was further confirmed by phenotypic analysis of colonies by colony size (cell number) and immunophenotyping of luminal and basal markers Krt18 and Krt14, respectively. The largest difference in colony size was observed for the basal stem/progenitor cell subpopulation, where parity induced a decrease in colonies of ≥ 20 cells from 66% to 9% (Table 2A; Figure 5C). Considerably smaller parity-induced reductions in colony size were observed for the other epithelial cell subpopulations, although a substantial decrease was also seen for basal myoepithelial cells (Table 2A). With regard to Krt18/Krt14 double positivity, parity induced a reduction in Krt18/Krt14 double-positive colonies derived from basal stem/progenitor cells from 26% to 0 (Table 2A). In contrast, although Krt18/Krt14 double-positivity decreased by 27%, 57% of colonies derived from luminal Sca1$^-$ cells of parous mice maintained double positivity (Table 2A). No effects of parity on Krt18/Krt14 double positivity were observed for colonies derived from luminal Sca1$^+$ or basal myoepithelial cells (Table 2A), whereby myoepithelial cells did not give rise to double-positive colonies even when originating from virgin control mice. Thus, consistent with the basal mammary stem/progenitor cell subpopulation-specific reduction in the

Wnt/Notch signaling ratio, parity decreased colony-formation capacity, *in vitro* proliferation potential (colony number and size), and Krt18/Krt14 double positivity most prominently in the basal stem/progenitor cell subpopulation.

5.4.5 Parity decreases the *in vivo* reconstitution efficiency of the basal stem/progenitor cell subpopulation

To test their proliferation potential *in vivo*, we transplanted basal stem/progenitor cells into deepithelialized mammary glands ("cleared fat pads") [12]. It already was demonstrated that basal $CD49f^{High}$ cells have the highest mammary gland reconstitution ability of all mammary cell subpopulations in virgin mice [16,19]. Transplantation of FACS-isolated basal stem/progenitor cells into cleared fat pads demonstrated a significant decrease in the number of large outgrowths (≥10% and ≥25% of fat pad filled), indicating a decrease in the *in vivo* proliferation potential. Interestingly, when assessing also for rudimentary outgrowths (≥3% of fat pad filled), no significant difference in the number of outgrowths was observed between parous and virgin donors (Table 2B). Apart from the change in size, no qualitative differences were apparent between outgrowths from virgin and parous donors. In both cases, ductal as well as lobular structures were formed. Hence, early parity led to a reduction in *in vivo* mammary repopulating efficiency of FACS-isolated basal stem/progenitor cells, whereas their ability to differentiate into different mammary epithelial structures was maintained.

5.4.6 Decrease in the proportion of Wnt4-secreting cells after early parity can explain decreased Wnt signaling and reduced proliferation capacity in basal mammary stem/progenitor cells

Having observed most of the expected functional consequences of a decrease in the Wnt/Notch signaling ratio in basal stem/progenitor cells from parous mice, we finally

examined the possible cause of parity-induced reduction in canonical Wnt signaling and proliferation capacity in basal stem/progenitor cells. Because parity induced a greater than threefold decrease in Wnt ligand *Wnt4* gene expression (Figure 2A, B), and Wnt4 is known to be secreted in response to progesterone by hormone-sensing luminal cells [46], thus inducing canonical Wnt signaling in mammary stem/progenitor cells [47], a parity-induced decrease in estrogen/progesterone-sensitive luminal cells could explain the overall decrease in Wnt signaling in mammary stem cells. This hypothesis is supported by the reduction in the proportion of luminal $Sca1^+$ cells isolated from parous mice (Figure 1D) and by the demonstration that luminal $Sca1^+$ cells are hormone receptor positive (Additional file 6) [17]. Furthermore, immunohistochemical analysis of mammary gland sections for estrogen receptor alpha (ERα) and its target progesterone receptor (PR) showed a twofold decrease in ERα- and a threefold decrease in PR-positive cells in parous compared with age-matched virgin control mice (Figure 6A, B). These results were additionally verified by qPCR in total mammary cell suspensions (Figure 6C). Notably, expression of the luminal marker *Krt8* was similar in cell suspensions from parous and age-matched virgin control mice, supporting the conclusion of a specific decrease in hormone receptor-positive cells rather than a general cell loss after pregnancy. Furthermore, parity-induced reduction in progesterone-stimulated *Wnt4* expression was independent of blood progesterone, because average blood progesterone concentrations were similar in parous mice and age-matched virgin control mice in estrus (see Additional file 9). Finally, supplementation of the culture medium with recombinant Wnt4 stimulated *in vitro* proliferation capacity of basal myoepithelial and basal stem/progenitor cells from parous mice by +138% ± 22% and +140% ± 17%, respectively (Figure 7A). In contrast, no significant effects of recombinant Wnt4 on the colony-formation capacity of luminal $Sca1^-$ (+3.7% ± 3.0%) and luminal $Sca1^+$ cells from parous mice (-5.1% ± 2.9%) were observed. These data strongly suggest a causal relation between reduced number

of luminal progesterone receptor-positive/Wnt4-secreting cells and decreased Wnt/Notch signaling and proliferation potential of basal stem/progenitor cells after early pregnancy (Figure 7B).

In conclusion, all collected data indicate that early parity induces a decrease in luminal hormone-sensing ERα- and PR-positive cells, which leads to decreased *Wnt4* expression levels and to reduced Wnt signaling in basal stem/progenitor cells. Consistent with the decrease in the Wnt/Notch signaling ratio, differentiation is promoted and proliferation inhibited in basal stem/progenitor cells of parous mice.

5.5 Discussion

This study demonstrates that a history of early pregnancy changes the gene-expression profiles and functional properties of mammary epithelial cell subpopulations in a cell subtype-specific fashion. Most important, the following parity-induced alterations were observed in mice: (a) an induction of differentiation and downregulation of the Wnt/Notch signaling ratio in basal stem/progenitor cells; (b) a decrease in the *in vitro* and *in vivo* proliferation potential of isolated basal stem/progenitor cells; (c) a selective downregulation of potentially tumorigenic biofunctions in the basal stem/progenitor cell subpopulation; (d) a reduction in estrogen- and progesterone-responsive and Wnt4-secreting luminal cells; and (e) a rescue of the proliferation defect in basal stem/progenitor cells *in vitro* by recombinant Wnt4. The finding of a decreased Wnt/Notch signaling ratio provides direct experimental evidence for the hypothesis that early pregnancy changes the "genomic signature" of mammary stem/progenitor cells [7], causing their differentiation and reducing their proliferation potential. Furthermore, the data indicate a novel causal relation between parity-induced reduction in hormone-sensing and Wnt4-secreting luminal cells and altered biofunctions in basal stem/progenitor cells.

The basal $CD49f^{High}$ cells, as isolated in this study, are a subfraction of basal epithelial cells [19,21]. Basal $CD49f^{High}$ cells have been demonstrated previously to be enriched in mammary repopulating units (MRUs) (known as mammary stem cells (MaSCs)) [16,19] and to correspond to the Lin^{-} $CD24^{+}$ $CD29^{High}$ epithelial cell subpopulation isolated by an alternative method [18,19,45]. However, the isolated basal $CD49f^{High}$ epithelial cell subpopulation represents a heterogeneous cell fraction containing, in addition to MaSCs, basal progenitor cells and possibly mature myoepithelial cells [45]. Progenitor cells can be characterized *in vitro* by their colony-formation capacity [22,23], whereas MaSCs have

traditionally been defined by their *in vivo* regenerative capacity [16-18]. The observed effects of parity on the *in vitro* and *in vivo* proliferation capacity of the CD49f^{High} cell subpopulation (Table 2) suggests that both basal progenitor cells and basal MaSCs are the target of an early pregnancy within the mammary epithelium. Although the dramatic decrease in the *in vitro* proliferation capacity of the CD49f^{High} cell subpopulation (Figure 5) indicates a predominant effect of an early pregnancy on basal progenitor cells, the additional reduction in large *in vivo* outgrowths (≥10% of fat pad filled) and the prevalence of rudimentary outgrowths (≥3% of fat pad filled) after parity (Table 2) suggest that isolated basal MaSCs are also affected by an early pregnancy. Because basal mammary stem and progenitor cells are closely related and likely to be interdependent in their proliferation potentials, our data do not permit a definite discrimination between basal stem and progenitor cells as primary targets of pregnancy. Therefore, we adhered to the combined term basal stem/progenitor cells throughout this study.

We found the p53-p21 pathway to be enriched to a similar extent in all mammary epithelial cell subpopulations (Additional file 7) [39], and hence, although parity-caused induction of the p53-p21 pathway may explain the relatively modest decrease in *in vitro* colony-formation potential of the luminal Sca1^{+} cell subpopulation (Figure 5), it may contribute but cannot account for the almost complete proliferation block in basal stem/progenitor cells. The most prominent parity-induced alterations in gene expression in basal stem/progenitor cells were downregulation of the Wnt-signaling pathway, upregulation of the Notch-signaling pathway, and upregulation of differentiation genes. Decreased Wnt signaling in basal stem/progenitor cells from parous mice was verified on the protein level by measuring versican and nuclear β-catenin expression (Figures 3D and 4B, C). Wnt signaling has been shown to promote long-term expansion of cultured Lin^{-} CD24^{+} CD29^{High} cells and to provide a competitive

advantage in mammary gland reconstitution assays [43]. The latter is especially true for the expression of the classic Wnt target *Lgr5* [48], which was found in our study to be downregulated in the basal stem/progenitor cell subpopulation after parity. Notably, Wnt signaling inhibition was demonstrated to have an antiproliferative effect in $CD29^{High}$ cells [43]. Furthermore, Notch signaling was observed to reduce *in vitro* and *in vivo* proliferation of $CD29^{High}$ cells while promoting their differentiation [44,49]. Because the differentiation processes of stem and progenitor cells in many organs and in several model systems are dependent on the Wnt/Notch signaling ratio [50], an overall reduction in Wnt/Notch signaling ratio would be expected to have a dramatic antiproliferation and prodifferentiation effect in mammary basal stem/progenitor cells. This is exactly what we observed in the $CD49f^{High}$ cell subpopulation of parous mice. Thereby, the overall conclusion of our study is strengthened by the fact that all assays used (that is, transcriptome analysis, bioinformatics transcription factor, and gene-enrichment analyses, *in vitro* colony-forming assay, *in vivo* transplantation assay, and immunohistochemistry) pointed into the same direction. Thus, analysis of specific mammary epithelial cell subpopulations allowed the discovery of a decrease in the Wnt/Notch signaling ratio, which so far is the only plausible explanation for the observed differentiation burst and dramatic proliferation block experienced by basal stem/progenitor cells of parous mice.

As possible explanations for the parity-induced decrease in Wnt signaling, we found a marked increase in the activity of the Wnt repressor TCF3 in basal stem/progenitor cells and a more than threefold reduction in expression of the secreted Wnt ligand *Wnt4* in total mammary cells from parous mice. The latter corresponded to a similar decrease in Wnt4-secreting [46] and estrogen and progesterone receptor-positive luminal cells. Notably, a similar decrease in progesterone receptor α-positive cells after parity has also been observed

in human breast epithelium [51]. Hence, mechanistically, early parity decreases the hormone responsiveness of the mammary gland in mice by decreasing the number of estrogen/progesterone receptor-positive luminal cells. This reduces the paracrine signaling cascade mediated by Wnt4, inducing TCF3-dependent repression [52] and/or primary downregulation [47] of canonical Wnt signaling and secondary (reactive) upregulation of Notch signaling in basal stem/progenitor cells.

As a final consequence, proliferation is repressed and basal stem/progenitor cells differentiate. This mechanistic model is supported by the ability of recombinant Wnt4 to rescue the proliferation defect of basal stem/progenitor cells from parous mice *in vitro* (Figure 7).

Our findings in specific mammary epithelial cell subpopulations are in part consistent with and in part contradictory to studies in entire breasts/mammary glands, total mammary cells, or total mammary epithelial cells. With respect to the transcriptome analysis, our studies in total mammary cells agree with previous reports in the entire mammary glands [9,10]. However, in intact mammary glands or total mammary cells, the additional presence of stromal and dominant epithelial cell subtypes might mask the detection of key signaling-pathway changes. Indeed, our study demonstrates that isolation of specific mammary epithelial cell subpopulations is a prerequisite for the detection of a decrease in Wnt/Notch signaling ratio in basal stem/progenitor cells. A similar masking effect by stromal and dominant epithelial cell subtypes (for example, strong clonogenic luminal Sca1$^-$ cells) might also explain why a previous study with a similar early-pregnancy protocol did not observe a parity-induced reduction in *in vitro* proliferation of total mixed mammary cells [20]. Controversial results have also been reported with respect to the effect of parity on the *in vivo* mammary-repopulating capacity. Hence, although Britt *et al*. [21] found no effects of late

pregnancy (9 weeks) on mammary-repopulating units (MRUs) in total mammary epithelial cells, Siwko *et al.* [20] (early-pregnancy protocol) observed a parity-induced reduction in the mammary-repopulating capacity of total mixed mammary cells. With a similar early-pregnancy protocol and the same cut-off for mammary gland outgrowth (≥10% of fat pad filled), our findings in isolated mammary basal stem/progenitor cells appear consistent with the observations of Siwko *et al*. However, given that our studies were performed with isolated mammary basal stem/progenitor cell subpopulations, our findings are not directly comparable with and neither confirm nor contradict previous studies using total mammary (epithelial) cells [20,21]. Furthermore, the fact that the number of smaller outgrowths (≥3% of fat pad filled) was unchanged after parity suggests that MRUs survive after pregnancy despite their reduced reconstitution efficiency. This conclusion is in line with the recent demonstration that Wnt-responsive mammary epithelial stem cells persist after parity in *Axin2* reporter mice [15].

It is intriguing to speculate that marked growth inhibitory effects and the downregulation of canonical Wnt signaling in basal stem/progenitor cells account, at least in part, for the cancer-protective effect of early pregnancy. Increases in canonical Wnt signaling have been linked repeatedly to oncogenesis [53,54]. Moreover, downregulation of the Wnt inhibitory protein Sfrp1 and overexpression of the Wnt target versican have been associated with carcinogenesis [55,56]. In the transcriptome analysis reported here, *Sfrp1* was upregulated and versican downregulated, thus supporting a parity-induced anticarcinogenic effect. Moreover, Li *et al*. [57] showed that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Hence, the contribution of decreased canonical Wnt signaling in basal stem/progenitor cells to the cancer-protective effect of early pregnancy may be in conjunction with other tumor-

suppressing mechanisms, such as parity-induced induction of p53 [58,59]. Thereby, the increase in the TCF3 repressor activity in basal stem/progenitor cells is expected to elevate the threshold further for the activation of tumorigenic Wnt signaling [52]. In addition to decreased Wnt signaling in basal stem/progenitor cells, decreased ERα- and PR-positive cells could also be a mechanism for the breast cancer-protective effect of an early pregnancy. This is especially relevant, given the specific protective effect of pregnancy against ER/PR-positive tumors [60].

Furthermore, mammary epithelial cell differentiation *per se* has been suggested to exert a breast cancer-protective effect. This has been challenged, however, by the observation that differentiation-causing agents such as placental lactogen and perphenazine failed to protect against carcinogenesis in rodents [3,61]. Also, the hypothesis of a potential breast cancer-protective effect of mammary stromal cells [62] is not supported by our study, because stromal cells exhibited by far the fewest parity-induced gene-expression changes. However, our stromal cell subpopulation was not homogenous and devoid of immune cells, which may have masked some parity-induced alterations. In any case, the parity-induced downregulation of the Wnt/Notch signaling ratio in basal stem/progenitor cells represents a possible important mechanism for the breast cancer-protective effect of early pregnancy.

5.6 Conclusions

This study identified downregulation of the Wnt/Notch signaling ratio in basal stem/progenitor cells as the dominant early parity-induced alteration of gene expression in mice. This change in gene expression is specific for basal mammary stem/progenitor cells, is associated with proliferation defects *in vitro* and *in vivo,* and is probably caused by an early parity-induced decrease in hormone-sensitive and Wnt4-secreting luminal cells. Importantly, because a similar reduction in progesterone receptor α-positive luminal cells has been reported in women [51], parity-induced alterations in Wnt/Notch signaling pathways may also occur in human basal stem/progenitor cells. Testing whether Wnt inhibitors mimic early parity-induced breast cancer protection warrants further investigation.

5.7 Abbreviations

ER, estrogen receptor; FDR, false discovery rate; FWER, family-wise error rate; GSEA, gene-set enrichment analysis; MARA, motif activity response analysis; MaSC, mammary stem cell; MRU, mammary repopulating unit; NES, normalized enrichment score; PR, progesterone receptor; RBC, red blood cell; WBC, white blood cell.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FM-A conceived of the hypothesis, prepared the mice, designed and performed the experiments, and wrote the manuscript. EM introduced FM-A to the techniques and assisted with some of the experiments. TR oversaw the microarray studies and assisted in the analysis of the microarray data. HB provided technical assistance with the immunohistochemistry. SD provided technical and intellectual support. DM provided technical assistance with the transplantation experiments, and IK performed fat-pad clearings and cell injections. PB and EvN performed the MARA analysis and provided intellectual support. MB-A conceived of the hypothesis, directed the project, and revised the manuscript. All authors read and approved the final manuscript.

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5.9 Figure legends

Figure 1. The CD24/Sca1 and CD49f^{High}/CD24 flow-cytometry profiles of parous and age-matched virgin mice are similar. (A) Schematic illustration of the cell-isolation strategy and representative flow-cytometry pseudocolor plots of mammary cells from age-matched virgin control mice. After depletion of CD45^{+} white blood cells, luminal and basal mammary epithelial cells were separated on the basis of CD24 and Sca1 expression. Further separation of basal cells into myoepithelial and basal stem/progenitor cell subpopulations was based on the expression of CD24 and CD49f. The isolated mammary epithelial cell subpopulations included luminal Sca1^{+} (CD24^{+High}Sca1^{+}) cells, luminal Sca1^{-} (CD24^{+High}Sca1^{-}) cells, basal CD49f^{High} (CD24^{+Low}Sca1^{-}CD49f^{High}) or basal stem/progenitor cells, and basal myoepithelial (CD24^{+Low}Sca1^{-}CD49f^{Low}) cells. **(B)** Outline of the mouse mating, parturition, weaning, and involution protocol. **(C)** Representative flow-cytometry pseudocolor plots of mammary cells from parous mice. The gates applied were the same as those for age-matched virgin controls. **(D)** Bar graph showing the distribution of mammary epithelial cell subpopulations comparing cells from parous with age-matched virgin control mice. Data represent the mean ± SEM of seven cell-isolation experiments with a minimum of 10 mice per experiment. The proportion of luminal Sca1^{+} cells was reduced by approximately 50% in parous mice ($P = 0.02$ with a two-tailed unpaired Student *t* test).

Figure 2. Parity-induced gene signature in total mammary cell suspensions. (A) Heat-map and cluster analysis of differential gene expression in total isolated mammary cells from age-matched virgin control and parous mice. Gene expression is presented as normalized Z-scores, defined as $Z = (x-\mu)/sd$ to allow visualization. A cut-off of $P < 0.05$ was applied. If multiple probe sets existed for the same gene, the probe set with the largest change in expression was selected. Four independent experiments were performed with 10 mice (five

virgins; five parous) per experiment. **(B)** qPCR validation of pregnancy-induced gene-expression changes in total mammary cell suspensions. Fold changes are shown in relation to expression in cell suspensions from age-matched virgin control mice. Ct values were normalized to the reference gene *Hprt*. As control for luminal epithelial cell number, qPCR for *Krt8* was performed. Data represent the mean ± SEM of four independent experiments with 10 mice (five virgins; five parous) per experiment.

Figure 3. Parity leads to differentiation and decreases the Wnt/Notch signaling ratio in basal stem/progenitor cells. (A) Bar graph depicting the number of gene-expression changes in FACS-sorted mammary stromal and epithelial cell subpopulations from parous mice compared with age-matched virgin control mice by using a cut-off of fold change >1.5 and an adjusted *P* value <0.05. By far the most gene-expression changes were observed in basal stem/progenitor cells. Three independent experiments were performed with 10 mice (five virgins; five parous) per experiment. **(B)** Schematic illustration of prominent gene-expression changes in FACS-sorted basal stem/progenitor cells from parous as compared with age-matched virgin control mice. Fold changes are shown in parentheses with upregulated genes denoted as positive (+), and downregulated genes, as negative (-). Differentiation genes were upregulated (blue), Wnt target genes were downregulated (green), Wnt inhibitor *Sfrp1* was upregulated (green) and overall Notch signaling (orange) was increased in basal stem/progenitor cells from parous mice. **(C)** qPCR validation of the changes in gene expression in basal stem/progenitor cells of parous mice. All classic Wnt target genes were downregulated, including *Lgr5*, *Axin2*, and versican (*Vcan*), whereas the more ubiquitously regulated target *Myc* was unchanged. In all cases, fold changes are shown relative to cells from age-matched virgin control mice. Ct values were normalized to the reference genes *Hprt* and *Ubc* [63]. Data represent the mean ± SEM of three independent experiments with 10

mice (five virgins; five parous) per experiment. **(D)** Representative images and quantification of immunostaining for the Wnt target gene versican in mammary gland sections from age-matched virgin and parous mice in estrus. Scale bar, 50 μm. Quantitative data represent the mean ± SEM from 60 randomly selected images from three virgin and three parous mice.

Figure 4. Effects of parity on Wnt transcription-factor activities and nuclear β-catenin in basal mammary epithelial cells. (A) Wnt transcription factor LEF1/TCF7 motif activity in basal stem/progenitor cells from parous as compared with virgin control mice, as predicted by MARA [32]. The binding motif of the LEF1/TCF7 transcription factor is shown in color. **(B)** Representative images of immunostaining for β-catenin in mammary gland sections from age-matched virgin and parous mice in estrus. Arrow, basal mammary epithelial cells with nuclear β-catenin. Arrowhead, basal mammary epithelial cells lacking nuclear β-catenin. Scale bar, 10 μm. **(C)** Bar graph representing the quantification of nuclear β-catenin in mammary gland sections of virgin and parous mice. Data represent the mean ± SD (virgin mice: $n = 3$; parous mice: $n = 3$). $P = 0.004$ with the two-tailed unpaired Student t test. **(D)** Transcription-factor activities in basal stem/progenitor cells, predicted on the basis of target gene expression by Ingenuity IPA [40]. Activity is reported as Z scores (positive Z score, upregulation; negative Z score, downregulation) by using a cut-off of linear fold change >1.5 and P value <0.05. **(E)** BioFunctions most strongly downregulated (blue) or upregulated (orange) in basal stem/progenitor cells, as calculated by Ingenuity IPA [40]. The color of the fields represents the Z score; the size of the fields represents the number of genes. A stringent cut-off of linear fold change >2 and P value <0.01 was used to minimize the number of false positives.

Figure 5. Parity reduces the progenitor potential of mammary epithelial cell subpopulations. (A) Representative images of individual wells with colonies formed by the specified cell subpopulations from age-matched virgin and parous mice. Scale bar, 4 mm. **(B)** Bar graph comparing the colony-forming capacities of myoepithelial cells, basal stem/progenitor cells, luminal $Sca1^-$, and luminal $Sca1^+$ cells of age-matched virgin and parous mice. Data are from three independent experiments and represent the mean ± SEM of colonies per well; 18 wells were assessed per cell type. *$P < 0.015$, NS: not significant ($P = 0.08$), by using two-tailed unpaired Student *t* test. **(C)** Representative images for the immunophenotyping of 5-day-old colonies grown from myoepithelial cells, basal stem/progenitor cells, luminal $Sca1^-$, and luminal $Sca1^+$ cells. The colonies were stained for luminal Krt18 (green) and basal Krt14 (red) expression. Hoechst 33342 (blue) was used to distinguish nuclei and to label feeder cells (negative control). Scale bar, 200 μm.

Figure 6. Early pregnancy decreases the proportion of progesterone receptor (PR) and estrogen receptor α (ERα)-positive cells. (A) Representative images of immunostaining for PR and ERα in mammary gland sections from age-matched virgin and parous mice in estrus. Scale bar, 50 μm. **(B)** Bar graph comparing the relative frequency of estrogen- and progesterone receptor-positive cells between mammary glands of virgin and parous mice. Data represent the mean ± SD (virgin mice, $n = 6$; parous mice, $n = 5$). For PR, $P = 3.70E-07$; for ERα, $P = 0.003$, by using two-tailed unpaired Student *t* test. **(C)** qPCR for progesterone receptor (*Pgr*), estrogen receptor alpha (*Esr1*), and the luminal marker keratin 8 (*Krt8*) genes in total mammary cell suspensions. Data are expressed as the mean ± SEM from four groups of a minimum of five parous and five age-matched virgin control mice.

Figure 7. Effect of Wnt4 on the proliferation capacity of basal stem/progenitor cells after early pregnancy in mice. (A) Recombinant Wnt4 rescues the parity-induced *in vitro* proliferation defect in basal mammary epithelial cells. Selected mammary epithelial cells from parous mice were cultured in the absence or presence of recombinant Wnt4. Three independent experiments were performed. Data represent the mean ± SEM of colonies per well, with six to nine wells assessed per cell type. $*P \leq 0.02$ (two-tailed unpaired Student *t* test). **(B)** Mechanistic model illustrating the parity-induced decrease in hormone-sensing and Wnt4-secreting luminal cells on the Wnt/Notch signaling pathways and the proliferation/differentiation potential in basal stem/progenitor cells.

Figure 1 Meier-Abt et al.

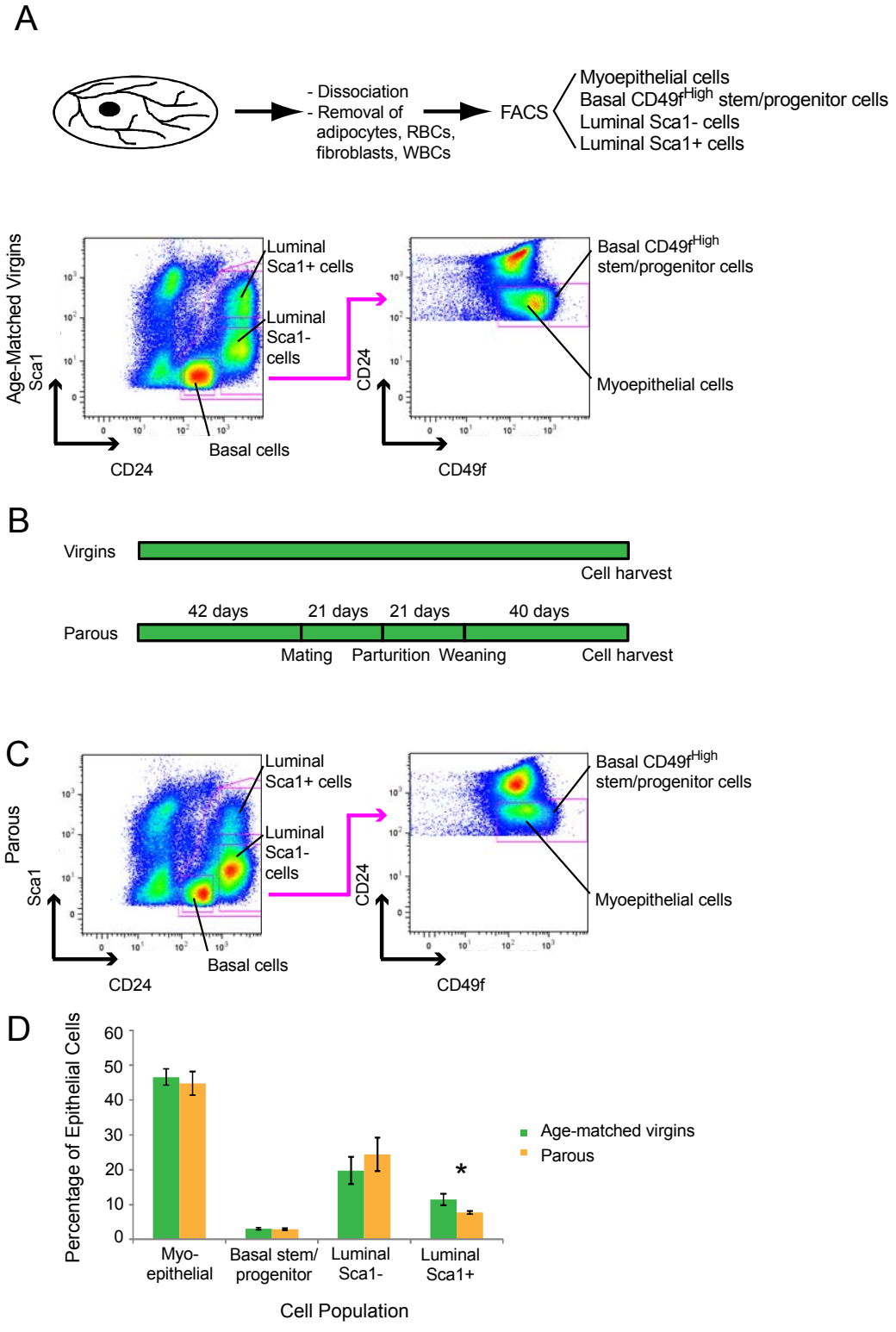


Figure 2 Meier-Abt et al.

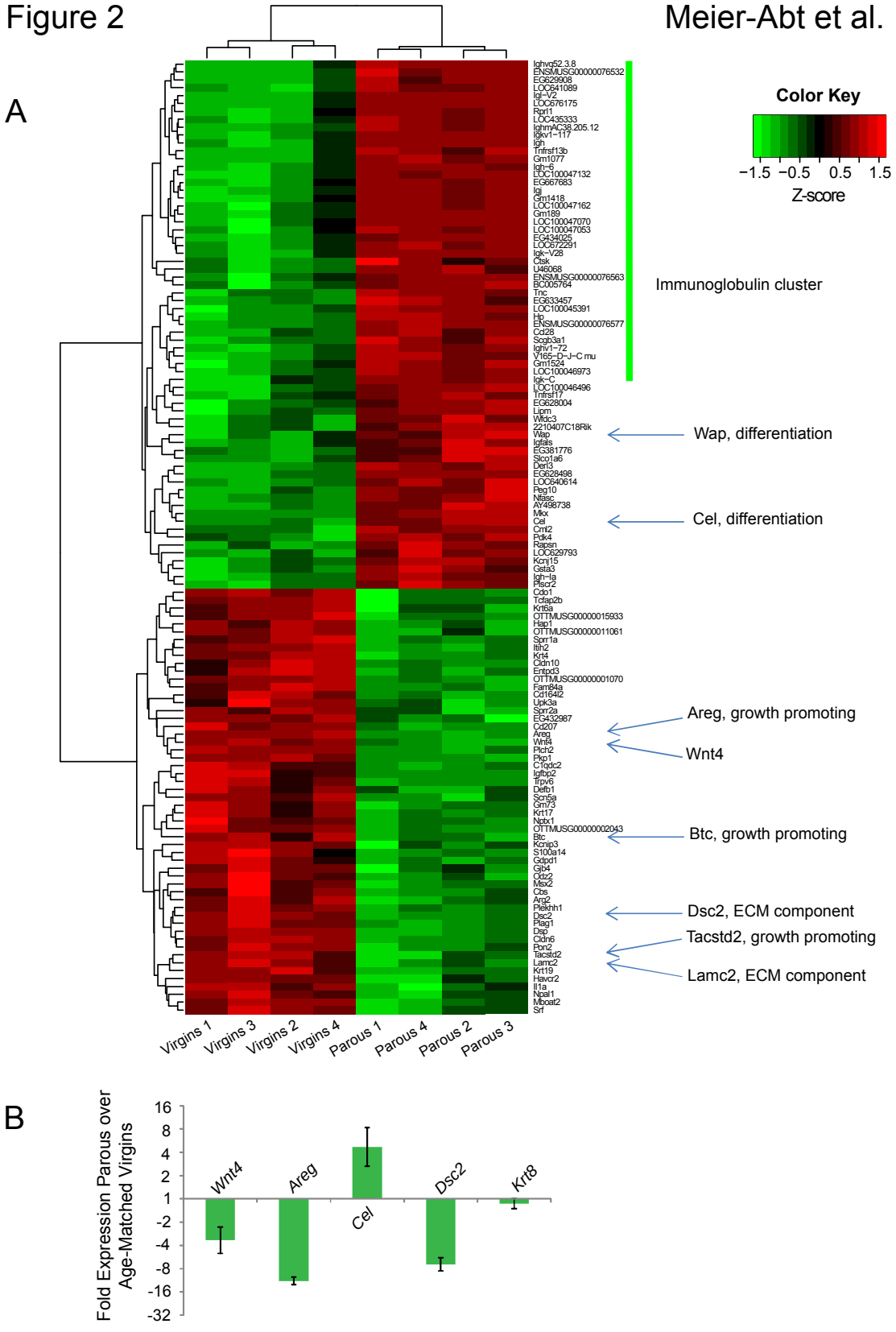
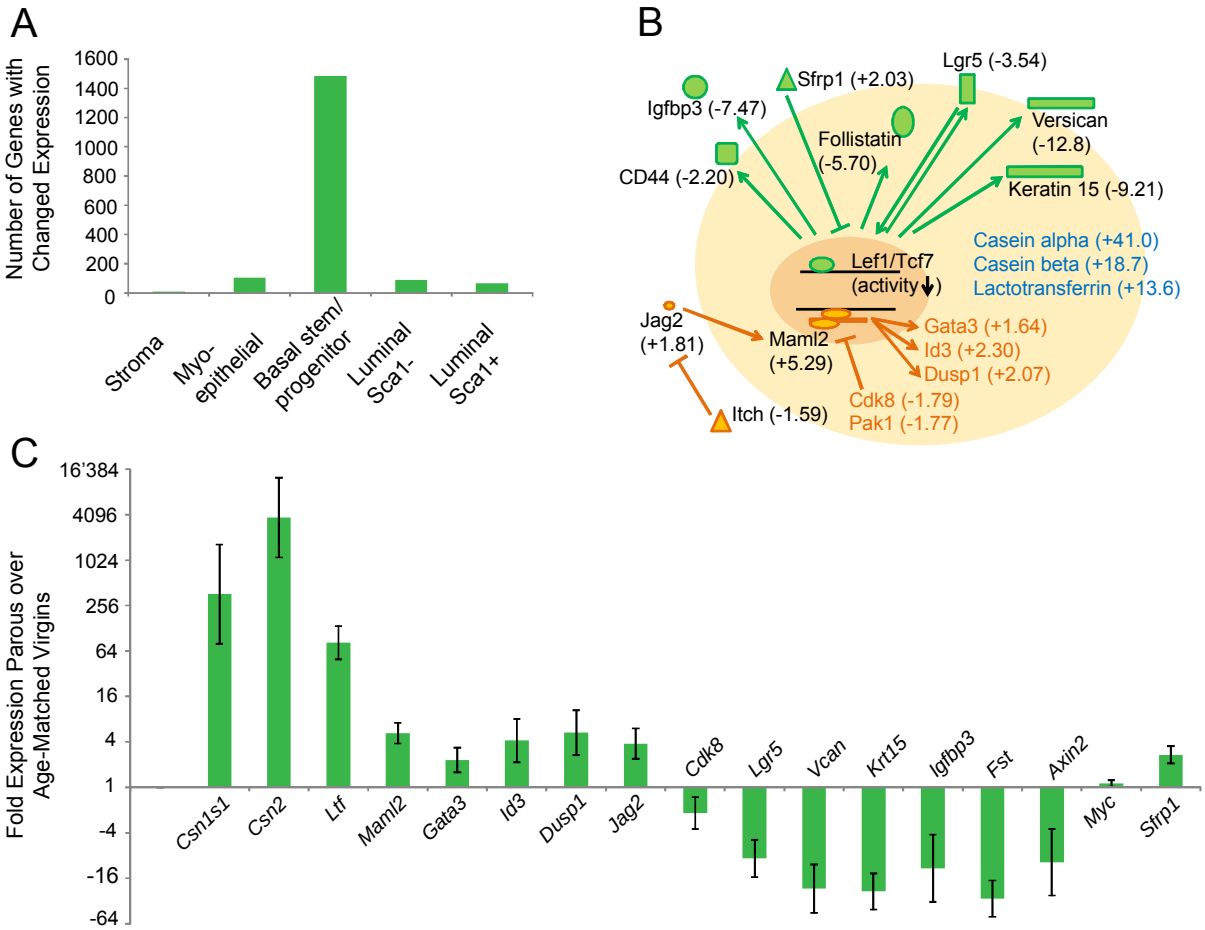
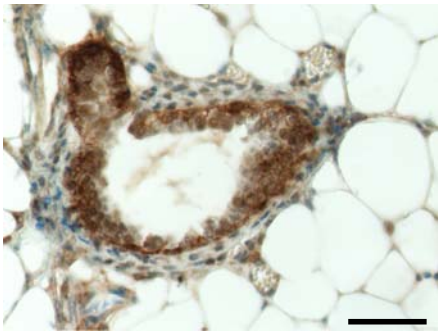


Figure 3 Meier-Abt et al.

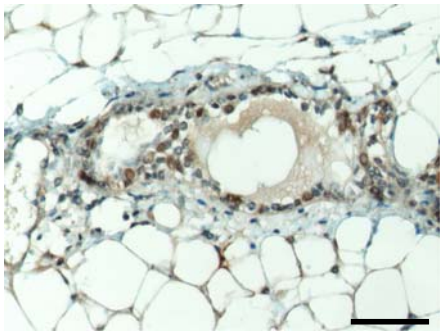


D

Versican Virgins



Versican Parous



Mean number of versican (brown) pixels per image:

Virgins: 38'700 ± 6954

Parous: 127 ± 33

P=5.48E-07

Figure 4 Meier-Abt et al.

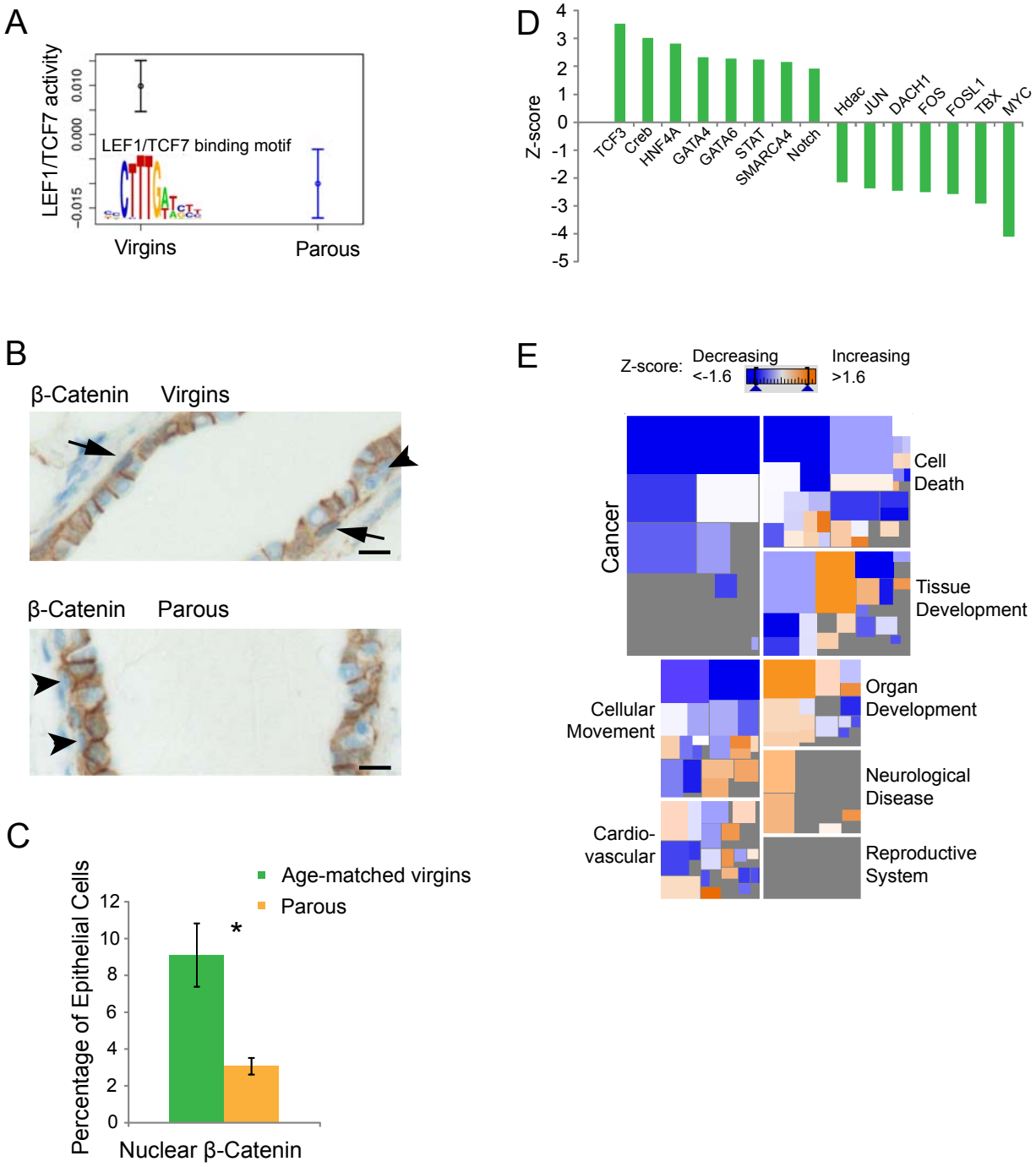


Figure 5 Meier-Abt et al.

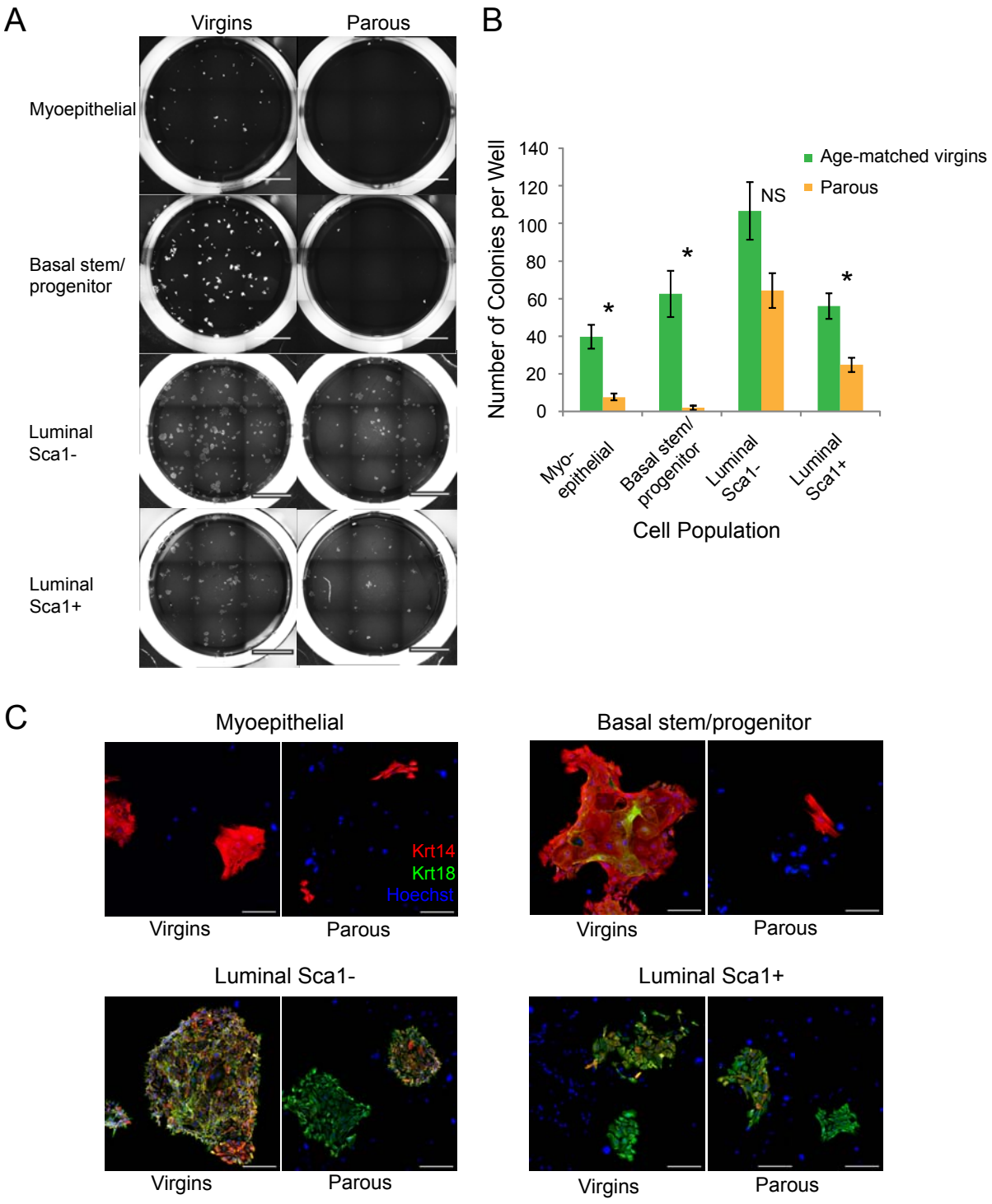


Figure 6 Meier-Abt et al.

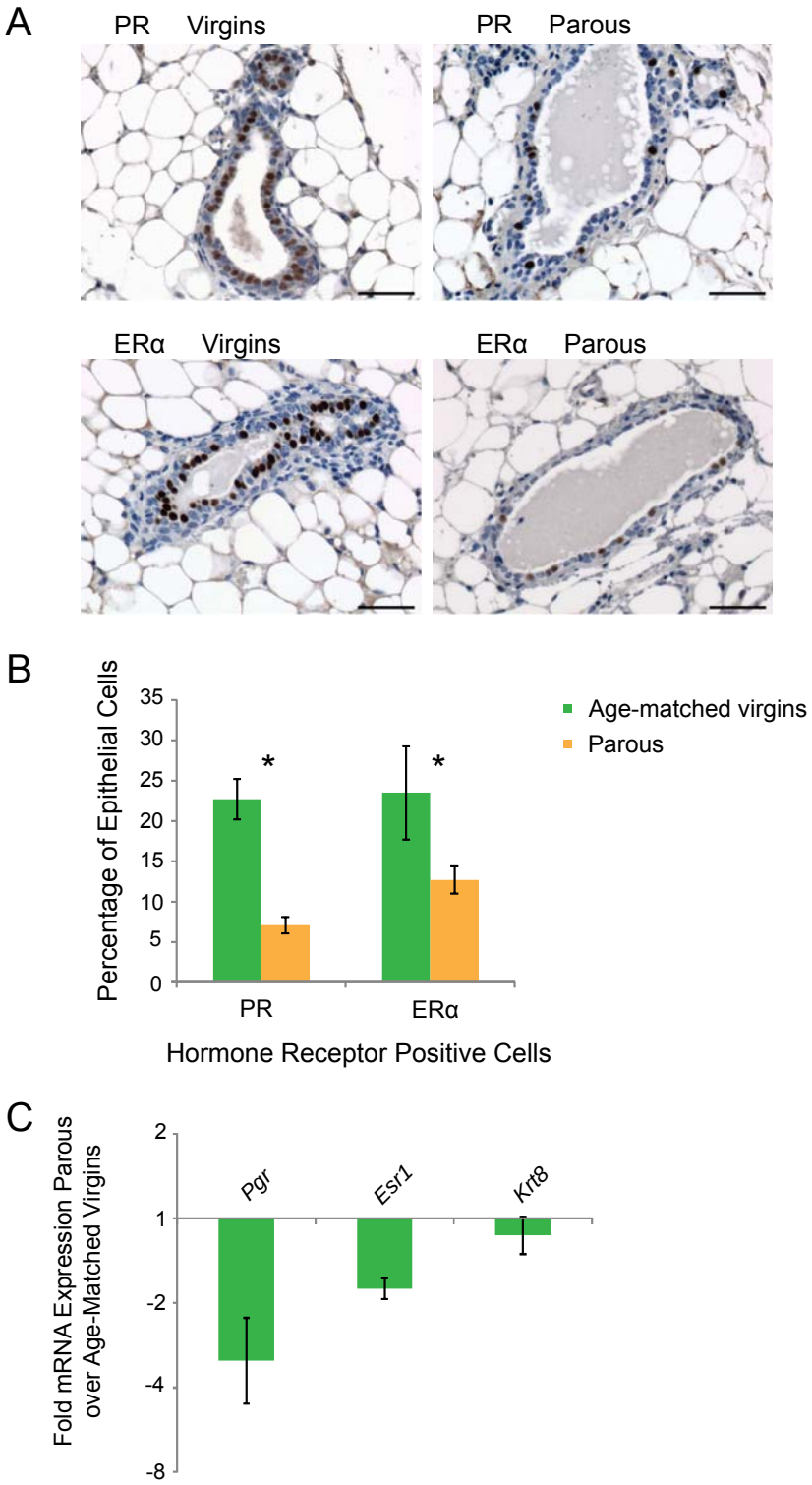
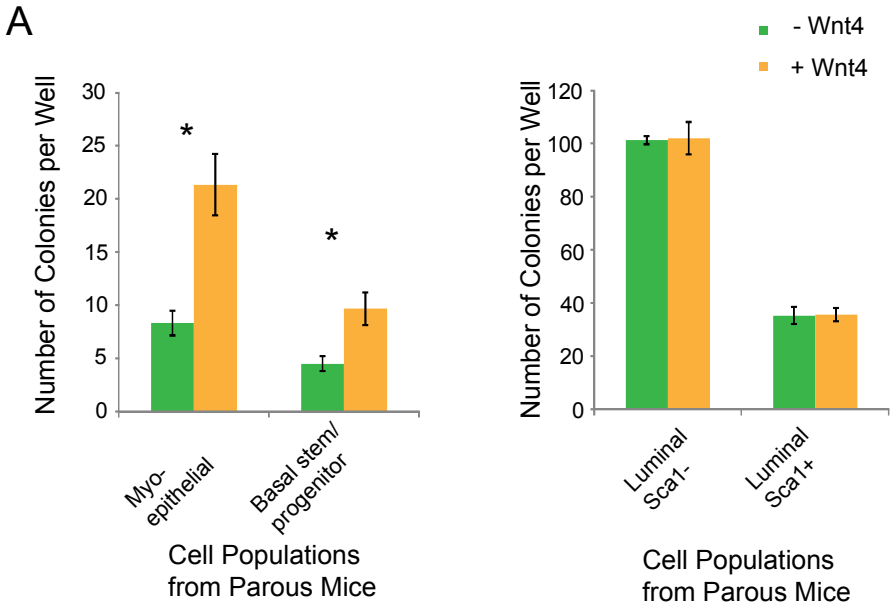
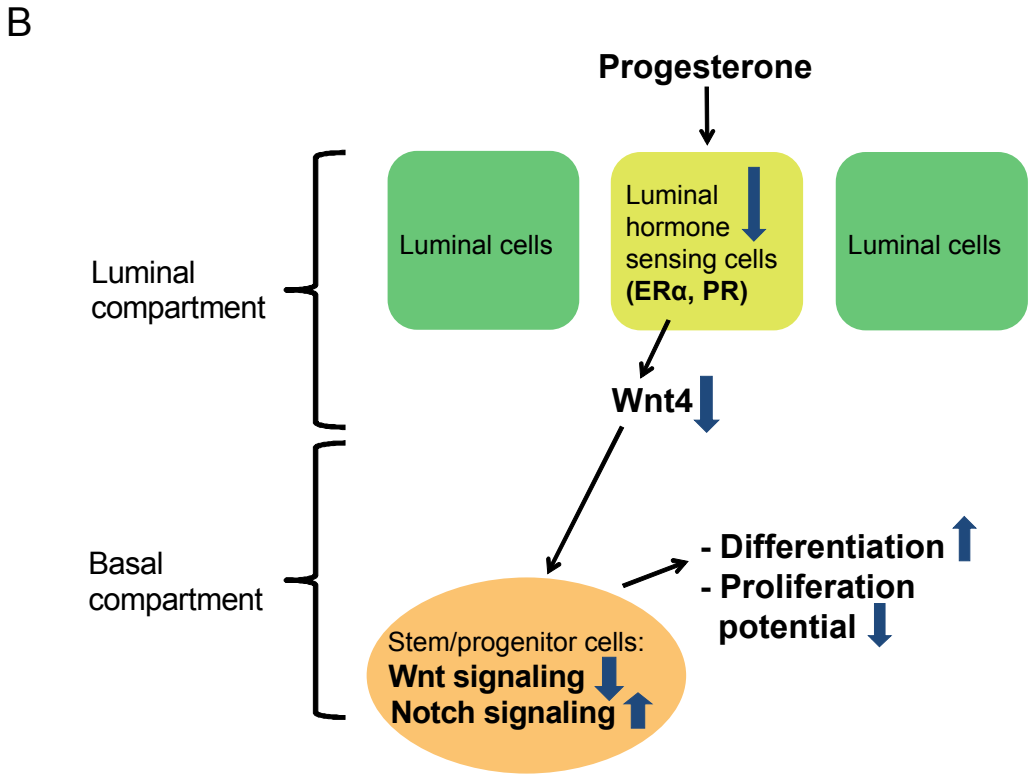


Figure 7 Meier-Abt et al.





5.10 Tables

Table 1: Wnt signaling is decreased and Notch signaling is upregulated in basal stem/progenitor cells

	Wnt signaling				**Notch signaling**			
	NES	*P*-value	FDR	FWER	NES	*P*-value	FDR	FWER
Myoepithelial	-1.47	0.03	0.06	0.04	1.08	0.35	0.34	0.74
Basal stem/progenitor	**-1.70**	**<0.01**	**<0.01**	**<0.01**	**1.64**	**<0.01**	**0.02**	**0.01**
Luminal Sca1-	-1.08	0.33	0.64	0.37	1.08	0.32	0.62	0.45
Luminal Sca1+	-0.68	0.99	0.99	0.74	0.83	0.77	1.00	0.93

Gene set enrichment analysis (GSEA) for Wnt and Notch signaling as defined by the expression of Wnt or Notch target genes (see Methods) showed a decrease in the Wnt/Notch signaling ratio (strongly negative/positive normalized enrichment scores (NES)), which was specific for the basal stem/progenitor cells. Significance was determined by a cut-off of a nominal *P*-value <0.01, a false discovery rate (FDR) <0.05 and a family-wise error rate (FWER) <0.05 as suggested by the GSEA homepage [28]. The gene set for Wnt signaling consisted of the reported Wnt targets in mammalian systems (derived from the Stanford Wnt homepage) and is depicted in Additional file 3. The gene set for Notch signaling was derived from the Broad Institute v3.0 and is termed Nguyen Notch1 targets. The number of permutations was set to 1000 and the permutation type was set to 'gene set', since there were less than 7 samples per phenotype [28]. Otherwise, the default settings were used.

Table 2: Functional characterization of isolated epithelial cell subpopulations

A	Fraction of large colonies		Fraction of double positive colonies	
	Virgins	Parous	Virgins	Parous
Myoepithelial[a]	32.7±7.4%[a]	9.6±2.3%[a]	0%	0%
Basal stem/progenitor[a]	66.2±7.2%[a]	9.3±4.5%[a]	26.1±2.7%	0%
Luminal Sca1-[b]	36.3±5.6%[b]	31.3±2.6%[b]	84.0±3.4%	57.1±2.9%
Luminal Sca1+[a]	73.3±1.8%[a]	71.8±4.1%[a]	64.1±3.6%	59.8±2.7%
[a]colony size ≥ 20 cells; [b]colony size ≥ 100 cells				

B, Number of basal stem/progenitor cells injected per cleared fat pad	Number of positive outgrowths[c,d,e]	
	Age-matched virgin control donors	Parous donors
1000	[c]8/9 [d]8/9 [e]8/9	[c]3/9 [d]3/9 [e]4/9
500	[c]8/11 [d]8/11 [e]8/11	[c]2/11 [d]2/11 [e]6/11
250	[c]2/6 [d]2/6 [e]2/6	[c]0/6 [d]1/6 [e]1/6
100	[c]0/6 [d]1/6 [e]1/6	[c]0/6 [d]0/6 [e]2/6
50	[c]0/7 [d]0/7 [e]0/7	[c]1/7 [d]1/7 [e]1/7
Repopulating frequency (95% confidence interval)	[c]1/507 (1/827 – 1/311) [d]1/472 (1/767 – 1/291) [e]1/472 (1/767 – 1/291)	[c]1/2468 (1/5522 – 1/1104) [d]1/2095 (1/4441 – 1/988) [e]1/907 (1/1582 – 1/520)
P value	[c]$P = 0.0004$; [d]$P = 0.0004$; [e]$P = 0.076$	
[c]positive outgrowth defined as ≥ 25% of fat pad filled; [d]positive outgrowth defined as ≥ 10% of fat pad filled; [e]positive outgrowth defined as ≥ 3% of fat pad filled		

A, Size and phenotype (Krt18/Krt14 double positivity) of colonies. At least 50 randomly selected colonies were examined for each group except for colonies derived from basal stem/progenitor cells of parous mice where all available colonies were used. Data represent the means ± SEM of three independent experiments with a minimum of 10 mice (5 virgins; 5 parous) per experiment.

B, Limiting dilution analysis of the repopulating capacity. The basal stem/progenitor cell subpopulations from parous (n=23) and age-matched virgin control (n=21) mice were injected into the cleared fat pads of 3-week-old syngeneic female recipient mice in limiting dilution numbers. Epithelial outgrowths were scored 8-9 weeks after injection. Number of positive outgrowths of specified sizes is indicated per number of mammary fat pads injected (i.e., X outgrowths per Y injections = X/Y). When analyzed statistically as previously described [18], a significant decrease in the number of large outgrowths was observed for parous donors.

5.11 Additional file legends

Additional file 1. Flow-cytometric separation of mammary epithelial cells from virgin control and parous mice. Representative flow-cytometry pseudocolor plots depicting the first steps in the gating strategy used to eliminate doublets, cell clumps, dead cells (DAPI bright) and white blood cells ($CD45^+$) during the procedure for isolating mammary epithelial cell subpopulations from virgin control **(A)** and parous **(B)** mice. Subsequent isolation steps are shown in Figure 1. **(C)** Bar graph showing the proportion of mammary epithelial cells relative to the total white blood cell depleted ($CD45^-$) mammary cells. The apparent modest decrease in total epithelial cells from parous mice was not significant ($P = 0.05$ by using two-tailed unpaired Student *t* test).

Additional file 2. Basal stem/progenitor cells from parous mice show reduced *in vivo* proliferation potential. Examples of an outgrowth (left) and of no outgrowth (right) from basal stem/progenitor cells of age-matched virgin control mice and of parous mice, respectively.

Additional file 3. Custom gene set of Wnt target genes. The gene set is composed of the Wnt targets that have been reported to be upregulated on canonical Wnt signaling in mammalian systems [64].

Additional file 4. Verification of luminal/basal origin and purity of isolated mammary epithelial cell subpopulations. (A) Immunofluorescent staining of isolated mammary epithelial cells with the luminal marker keratin 18 (Krt18) and the basal marker keratin 14 (Krt14). Basal myoepithelial cells were negative for Krt18 and positive for Krt14 in >95% of total cells. Conversely, luminal $Sca1^-$ and luminal $Sca1^+$ cells were positive for Krt18 and

negative for Krt14 in >95% of total cells. These data confirm the basal and luminal origin of the isolated cell subpopulations. Basal stem/progenitor cells were positive for Krt14 and Krt18 in >95% and about 20% of total cells, respectively. Data are representative of three independent experiments. Scale bar, 50 μm. (**B/C)** qPCR for *CD49f* and *Sca1* in FACS-sorted mammary epithelial cell subpopulations. Fold changes are shown relative to myoepithelial cells. Data are expressed as the mean ± SEM of three independent experiments.

Additional file 5. Control for complete involution. Representative images of whole mounts of mammary glands from virgins and parous mice 28 and 40 days after weaning. Mammary glands were completely involuted at 28 days and certainly at 40 days after weaning.

Additional file 6. Influence of cell number on transcriptome analysis and validation of the amplification method. (A) Pairwise correlation plot of transcriptome data derived from 2,000 and 50,000 myoepithelial and luminal $Sca1^{-}$ cells, and from 2,000 basal $CD49f^{High}$ stem/progenitor cells isolated from 11-week-old virgin mice (n = 6). Individual arrays were pairwise correlated by using the unfiltered data as input. Pearson correlation coefficients were calculated and mapped onto a gray scale from black (low values) to white (high values). Higher cell numbers resulted in higher reproducibility, as assessed by high Pearson correlation coefficients. However, although lower cell numbers resulted in lower Pearson correlation coefficients, all arrays of one cell subpopulation were clearly discernible from other subpopulations, irrespective of the cell number used. Thus, in the range of 2,000 to 50,000 cells, cell-subpopulation identity was more determining for cluster analyses than cell number. **(B)** qPCR on amplified cDNA of mammary epithelial cell subpopulations. Data were normalized to the reference gene *B2M* and are shown relative to 50,000 cells of myoepithelial cells. The basal marker keratin 14 (*Krt14*) was expressed by myoepithelial

cells and basal CD49f^{High} stem/progenitor cells, but not by luminal Sca1^{-} and luminal Sca1^{+} cells. Conversely, the luminal markers keratin 8 (*Krt8*) and keratin 19 (*Krt19*) were expressed by luminal Sca1^{-} and luminal Sca1^{+} cells, but not by myoepithelial and not by basal CD49f^{High} stem/progenitor cells. As expected, the estrogen receptor alpha (*Esr1*) was expressed by luminal Sca1^{+} cells only. Data represent the means of duplicates. **(C)** qPCR on unamplified cDNA. Data were processed and analyzed as in **(B)**. Changes in expression levels of the luminal and basal markers *Krt19* and *Krt14*, respectively, were similar to those of amplified cDNA, indicating that the amplification process was unbiased.

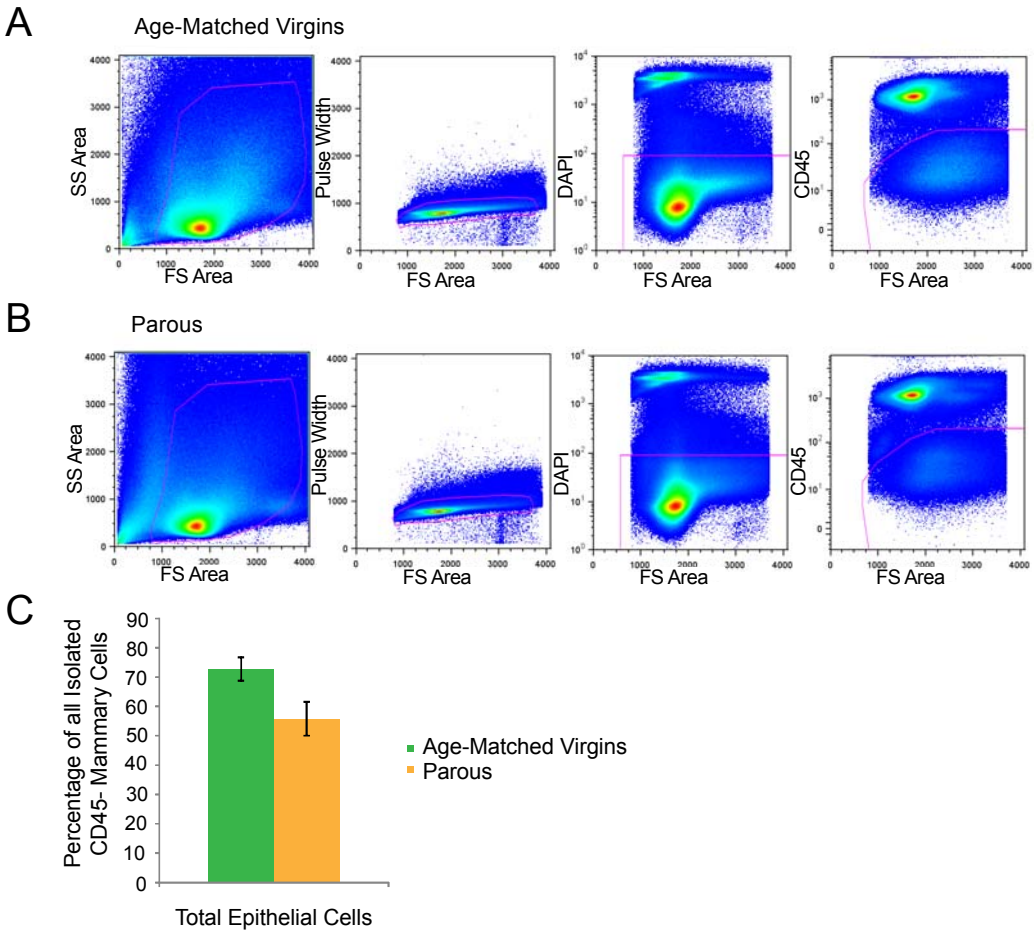
Additional file 7. The decrease in Wnt signaling is specific for basal stem/progenitor cells, whereas the p53-p21 pathway is upregulated to the same degree in all mammary epithelial cell subpopulations from parous mice. (A) Enrichment plots of Wnt target gene-set enrichment analysis [28,29] for isolated mammary epithelial cell subpopulations. The enrichment score is plotted against the ranked gene list, calculated by subtracting the gene expression levels of cells from age-matched virgins and parous mice. The gene set contained all canonical Wnt target genes reported in mammalian systems (Additional file 3). Thus, positive enrichment scores indicate an upregulation, and negative enrichment scores, a downregulation of Wnt signaling in cells of parous mice. Statistical analysis indicated specific downregulation of Wnt signaling in basal stem/progenitor cells from parous mice (Table 1). The apparent downregulation of Wnt signaling in myoepithelial cells, which are contaminated with basal stem/progenitor cells, was not significant. **(B)** Bar plot of gene-set enrichment analysis (GSEA)-calculated and normalized enrichment scores for the p53-p21 pathway previously identified by Sivaraman *et al*. [39]. The p53-p21 gene set was rendered by GSEA as significantly upregulated in all isolated mammary epithelial cell subpopulations from parous mice when testing for all signaling-pathway gene sets contained in v2.5 and

v3.0. The nominal *P* value, the false discovery rate (FDR), and the family-wise error rate (FWER) for this pathway were <0.01 for all mammary epithelial cell subpopulations; 1,000 permutations were performed with the permutation type "gene set." **(C)** Representative images of immunostaining for p21 and bar graph comparing the relative frequency of p21-positive epithelial cells in mammary gland sections from age-matched virgin and parous mice in estrus. Data represent the mean ± SD (virgin mice: $n = 3$; parous mice: $n = 3$). $P = 0.0007$, by using two-tailed unpaired Student *t* test. Scale bar, 25 μm.

Additional file 8. Twenty most significantly downregulated pathways in basal stem/progenitor cells after parity. The list was calculated by using v3.0 of GSEA [28]. The 1,000 permutations were performed by using the permutation type "gene set." In all other cases, the default settings were used.

Additional file 9. Blood progesterone concentrations in parous and age-matched virgin control mice in estrus do not change significantly. Plasma progesterone levels were measured with ELISA.

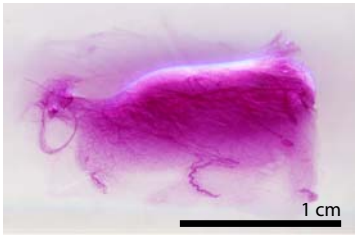
Additional file 1 Meier-Abt et al.



Additional file 2 Meier-Abt et al.

Outgrowth from virgin donors

No outgrowth from parous donors



Additional file 3

Custom gene set of Wnt target genes

MYC
CCND1
TCF7
LEF1
PPARD
JUN
FOSL1
PLAUR
MMP7
AXIN2
NRCAM
TCF4
GAST
CD44
CLDN1
BIRC5
VEGFA
FGF18
ATOH1
MET
EDN1
MYCBP
L1CAM
ID2
JAG1
TIAM1
NOS2
DKK1
FGF9
LBH
FGF20
LGR5
SOX17
RUNX2
GREM1
SALL4
CYR61
SOX2
PTTG
DLL1
FOXN1
MMP26
NANOG
POU5F1
SNAI1/2
FN1
FZD7
FST
WNT3A
ISL1
MMP2
MMP9
EN2
STRA6
EFNB1
ENPP2
ISLR
TWIST1
MMP3
T
GCG
CDX1
PTGS2
IRX3
SIX3
NEUROG1
SP5
NEUROD1
GBX2
WISP1
WISP2
IGF2
IGF1
VEGFC
IL6
CDX1
CDX4
SFRP2
PITX2
EGFR
EDA
KRT15
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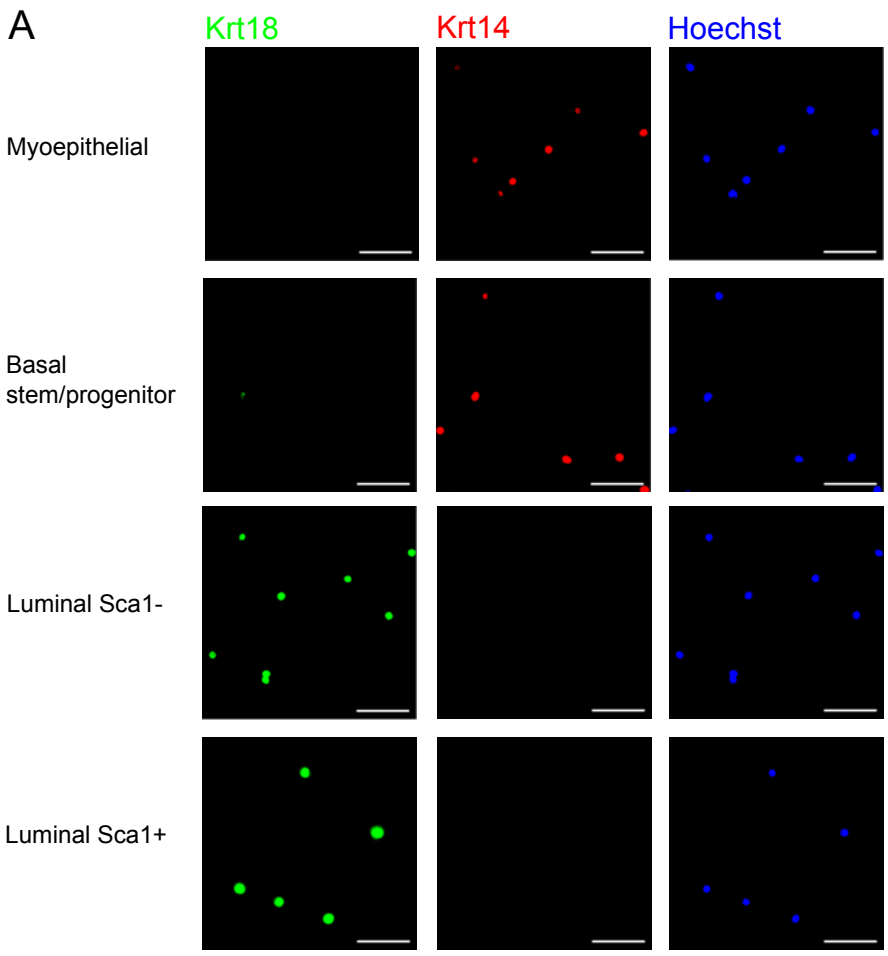
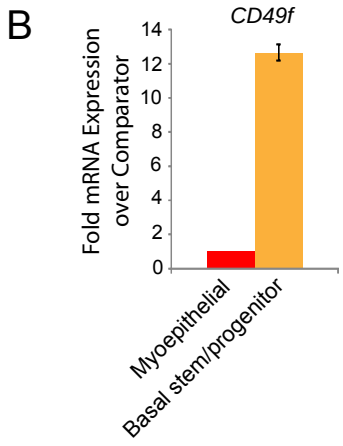
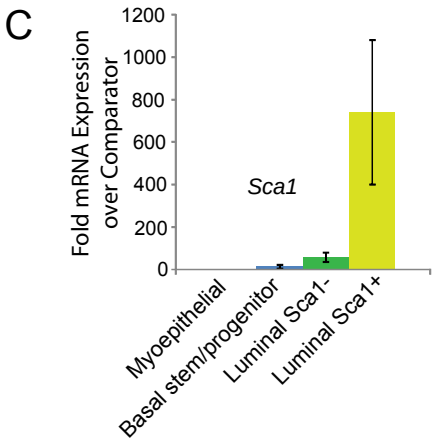
FGF4
IL8
RET
GJA1
VCAN
TNFRSF19
IGFBP3

Additional file 4 Meier-Abt et al.

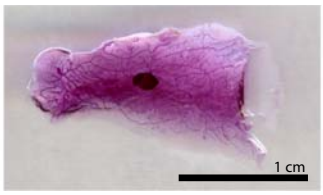




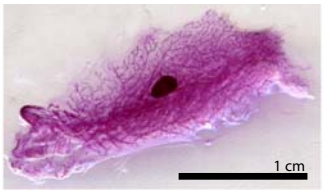


Additional file 5 Meier-Abt et al.

Virgin

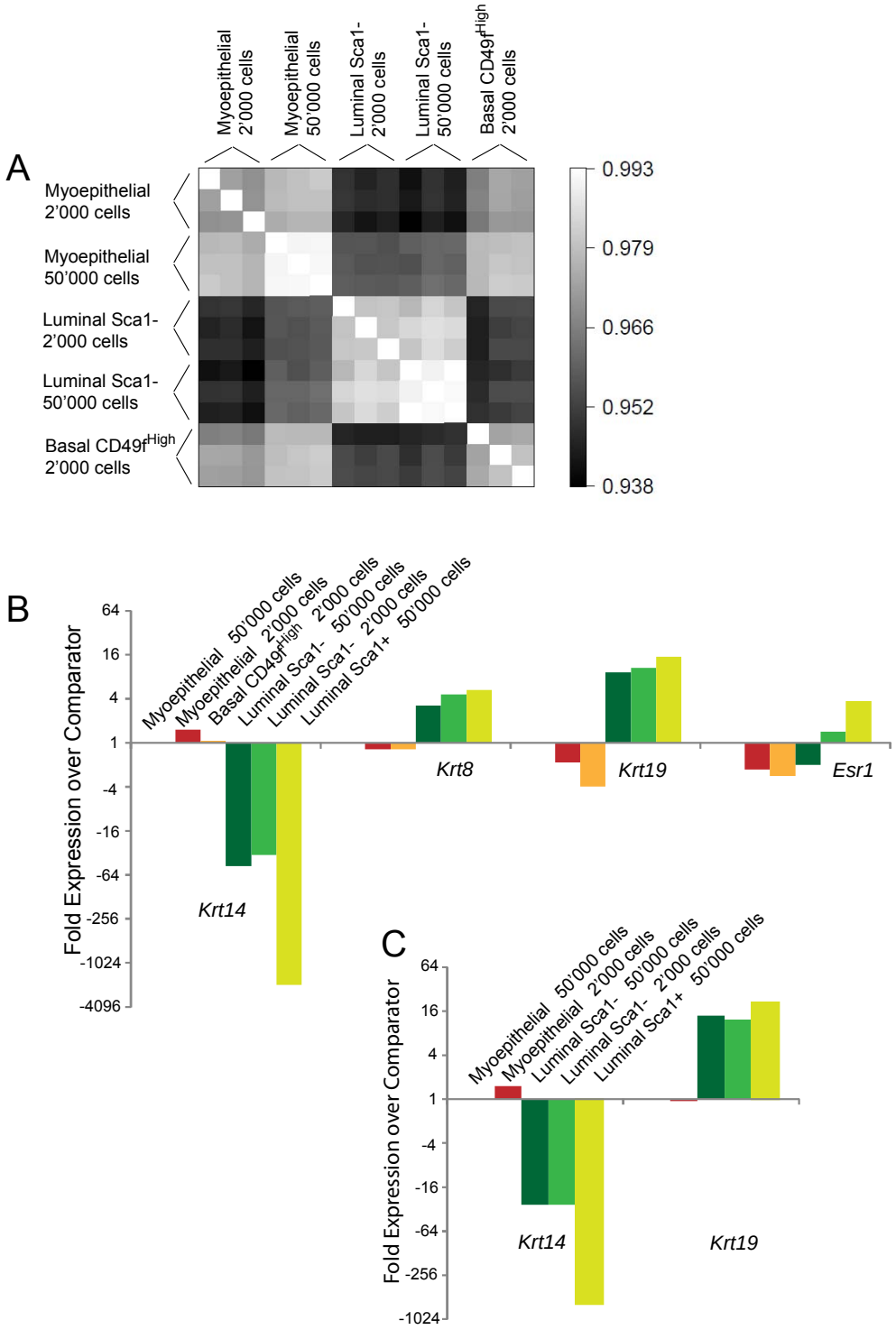


28 days post weaning

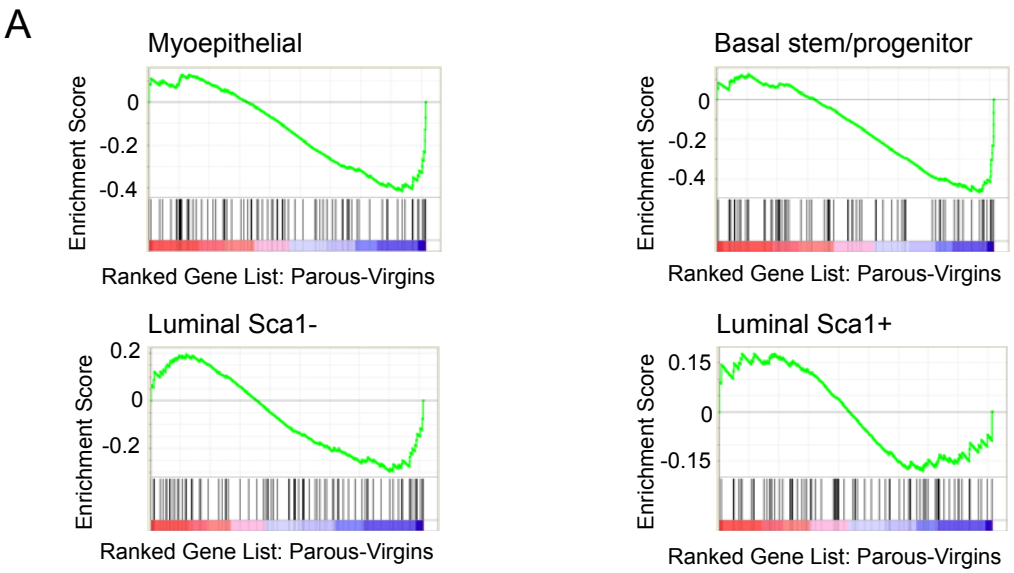


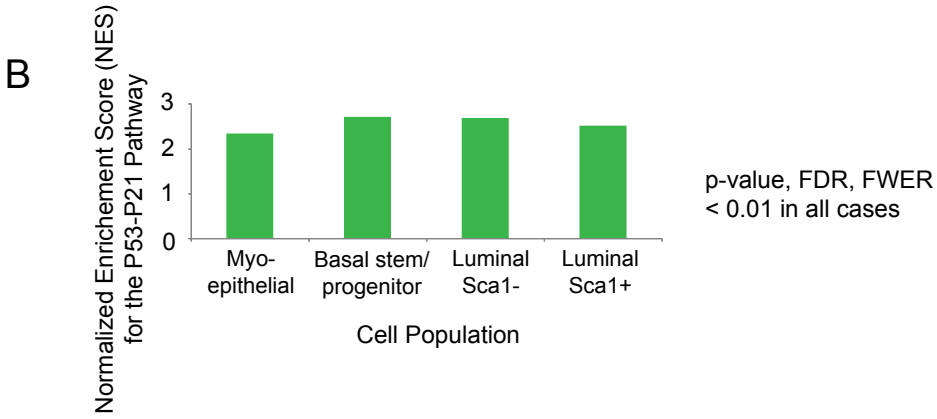
40 days post weaning

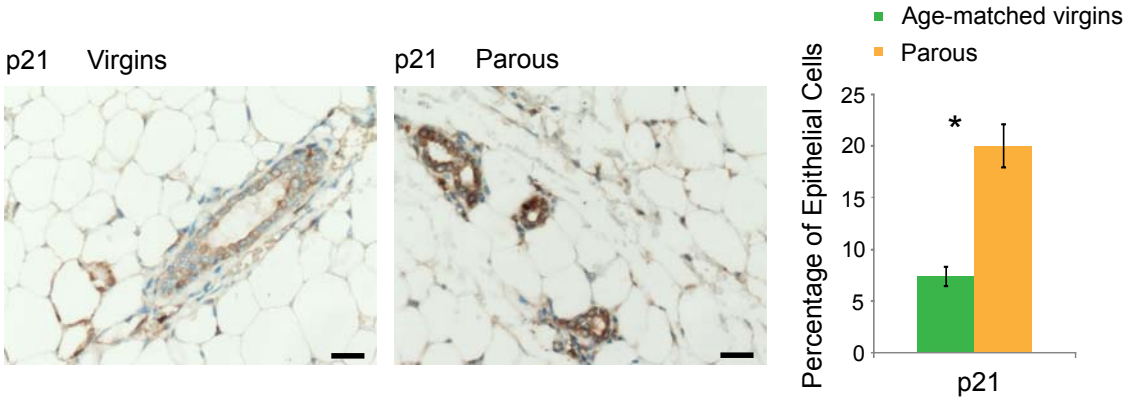
Additional file 6 Meier-Abt et al.



Additional file 7 Meier-Abt et al.







Additional file 8 Meier-Abt et al.

Twenty most significantly downregulated pathways in basal stem/progenitor cells after parity

Gene set name	NES	NOM p-value	FDR q-value	FWER p-value
KOBAYASHI_EGFR_SIGNALING_24HR_DN	-3.10156	<0.01	<0.01	<0.01
SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	-3.09002	<0.01	<0.01	<0.01
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	-3.03387	<0.01	<0.01	<0.01
SHEDDEN_LUNG_CANCER_POOR_SURVIVAL_A6	-2.91205	<0.01	<0.01	<0.01
HOFFMANN_LARGE_TO_SMALL_PRE_BII_LYMPHOCYTE_UP	-2.87939	<0.01	<0.01	<0.01
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_UP	-2.81022	<0.01	<0.01	<0.01
FURUKAWA_DUSP6_TARGETS_PCI35_DN	-2.7968	<0.01	<0.01	<0.01
ODONNELL_TFRC_TARGETS_DN	-2.77325	<0.01	<0.01	<0.01
WINNEPENNINCKX_MELANOMA_METASTASIS_UP	-2.7715	<0.01	<0.01	<0.01
CROONQUIST_IL6_DEPRIVATION_DN	-2.76799	<0.01	<0.01	<0.01
BENPORATH_PROLIFERATION	-2.71965	<0.01	<0.01	<0.01
WHITEFORD_PEDIATRIC_CANCER_MARKERS	-2.71767	<0.01	<0.01	<0.01
CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP	-2.71177	<0.01	<0.01	<0.01
LEE_EARLY_T_LYMPHOCYTE_UP	-2.70924	<0.01	<0.01	<0.01
GRAHAM_NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_DN	-2.69762	<0.01	<0.01	<0.01
KANG_DOXORUBICIN_RESISTANCE_UP	-2.6926	<0.01	<0.01	<0.01
FERREIRA_EWINGS_SARCOMA_UNSTABLE_VS_STABLE_UP	-2.6593	<0.01	<0.01	<0.01
BERENJENO_TRANSFORMED_BY_RHOA_UP	-2.64615	<0.01	<0.01	<0.01
PUJANA_BRCA2_PCC_NETWORK	-2.63882	<0.01	<0.01	<0.01
WILCOX_PRESPONSE_TO_PROGESTERONE_UP	-2.63688	<0.01	<0.01	<0.01

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Plasma progesterone levels in mice

	Average plasma progesterone concentration (ng/mL) ± s.d.
Age-matched virgin control mice in estrus (n=5)	**4.6 ± 1.8**
Parous mice in estrus (n=6)	**3.2 ± 0.9**
P value	**0.14**

6. GENERAL DISCUSSION

This study shows that early pregnancy does indeed induce cell autonomous processes that potentially can explain the observed breast cancer protective effect of early parity in rodents and in humans. More specifically, isolation of similar mammary epithelial cell subpopulations in parous and age-matched virgin control mice revealed that early pregnancy changes the "cell fates" of specific mammary epithelial cell subtypes by inducing cell subtype-specific alterations in gene expression profiles, proliferation capacities, and differentiation potentials. Most importantly, early pregnancy induced a decrease in the Wnt/Notch signaling ratio in the basal mammary stem/progenitor cell subpopulation. This alteration of cell fate determining signaling pathways was accompanied by a more differentiated phenotype and by a decrease in the *in vitro* proliferation and differentiation potentials. Whereas *in vivo* the proliferation potential of basal stem/progenitor cells was also reduced after early pregnancy, the differentiation potential remained unchanged. Furthermore, early pregnancy reduced the response of the mammary gland to progesterone signaling by decreasing the proportion of estrogen and progesterone receptor positive luminal cells, resulting in reduced expression of the progesterone target *Wnt4* despite constant progesterone plasma levels. Thereby, decreased *Wnt4* expression may well explain parity-induced reduction in Wnt signaling and associated proliferation failure in basal stem/progenitor cells, especially because recombinant Wnt4 rescued the parity-induced proliferation defects of this cell subpopulation. Hence, the findings suggest a direct link between reduced numbers of hormone receptor positive luminal cells and decreased Wnt signaling, reduced proliferation potential and increased differentiation phenotype of basal mammary stem and/or progenitor cells after pregnancy (see Figure 6.1).

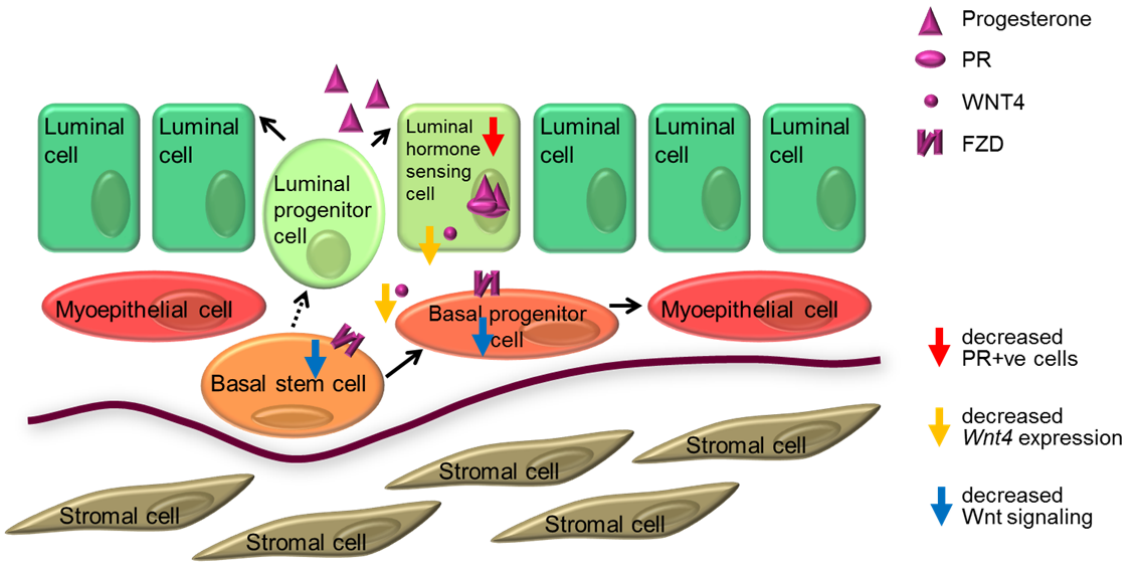

Figure 6.1 Schematic illustration of early pregnancy-induced cascade of molecular and cellular alterations in mouse mammary epithelial cell subpopulations

Early pregnancy decreases the proportion of progesterone receptor (PR) positive cells (red arrow), which decreases the expression of the Wnt ligand *Wnt4* (yellow arrow). This in turn reduces Wnt signaling in basal stem and/or progenitor cells (blue arrow), decreasing their proliferation potential and inducing a differentiated phenotype.

Although our data suggest a direct causal relation between decreased luminal Wnt4 secretion and decreased Wnt signaling and proliferation potential in basal stem and/or progenitor cells, the additional involvement of further Wnt ligands cannot be excluded. This relates especially to the inhibitor of canonical Wnt signaling Wnt5a. Notably, previous studies in total mammary glands have reported an increase in TGFβ signaling after pregnancy which in turn stimulates Wnt5a expression (Blakely et al., 2006; Blance et al., 2009; D'Cruz et al., 2002; Roarty and Serra, 2007). Wnt5a activates the planar cell polarity and/or the Wnt/calcium pathway, which in turn inhibit canonical Wnt signaling (Mikels and Nusse, 2006; Serra et al.,

2011; Topol et al., 2003; Westfall et al., 2003). Thus, activation of such non-canonical Wnt pathways could be involved in addition to reduced mammary *Wnt4* expression in the early pregnancy-induced decrease of canonical Wnt signaling in basal stem and/or progenitor cells.

Two anti-tumorigenic alterations in mammary glands of parous mice can potentially explain the breast cancer protective effect of early pregnancy. First, Wnt inhibition has been shown to have potent anti-proliferation and anti-cancer activity (Benad et al., 2011; Ettenberg et al., 2010; Hallett et al., 2012; King et al., 2012; Prosperi and Goss, 2010). In contrast, increased Wnt signaling and elevated proliferation potential in stem and progenitor cells are strongly linked to oncogenesis in the mammary gland (Li et al., 2003; Liu et al., 2004; Teuliere et al., 2005), and over 50% of women with breast cancer have activated canonical Wnt pathway promoting tumor progression (Benad et al., 2011; Lindvall et al., 2007). Second, based on the hypothesis that estrogen and progesterone receptor (ER/PR) positive breast cancers originate from ER/PR positive luminal mammary epithelial cells (Lim et al., 2009), the observed parity-induced reduction in the proportion of luminal hormone sensing cells (see Figure 6.1) could additionally contribute to decreased risk of breast tumorigenesis following pregnancy. Together with a decreased propensity of stem and progenitor cells to proliferate and generate new potential cells of tumor origin, the findings in this study are thus consistent with a breast cancer protective effect of early pregnancy. This is underscored by the decrease of potentially tumorigenic biofunctions as revealed by bioinformatics analyses (see 5.4.3). However, it cannot be excluded that besides the decreased functionality of the ER/PR-Wnt4-canonical Wnt signaling cascade and its subsequent proliferation defect in basal mammary stem and/or progenitor cells other anti-cancer mechanisms might also be involved in the reported breast cancer protective effect of an early pregnancy. Thus, the overall significance of Wnt inhibition and its molecular and cellular consequences for parity-induced breast cancer

protection remain to be demonstrated in long-term studies with synthetic Wnt inhibitors. Notably, several Wnt inhibitors are currently in development and at least two have entered Phase I trials (Curtin and Lorenzi, 2010).

The life-long nature of parity-induced breast cancer protection indicates the presence of possible epigenetic mechanisms downstream of reduced progesterone and/or Wnt signaling in specific mammary epithelial cell subtypes. This is especially relevant, because progesterone and Wnt signaling have been associated with epigenetic alterations including DNA methylation, as well as H3K27 and H3K4 trimethylation (Pal et al., 2013; Wohrle et al., 2007). Studies on potential parity-induced epigenetic changes in isolated mammary cell subpopulations are warranted.

Other open questions remain with respect to the time points of pregnancy and mammary cell analyses. In this study, special care was taken to induce pregnancy in mice as early as possible (i.e. 42 days). In addition, cell harvest was performed after complete involution had occurred (i.e. 40 days after weaning). Hence, the obtained results are related neither to the effect of late pregnancy nor to the duration of early parity-induced molecular and cellular alterations in mouse mammary epithelial cell subpopulations. Since early but not late pregnancy protects against breast cancer and this protective effect is of life-long duration, it would be especially interesting to investigate the age-dependent magnitude as well as the duration of parity-induced alterations. In this context, eight scenarios are theoretically possible. They are illustrated in Figure 6.2. However, not all scenarios are equally relevant, since the breast cancer protective effect of parity decreases with increasing age at first full-term pregnancy (see Figure 3.4.1). Whereas this age-related diminution of the breast cancer protective effect could be explained by the greater initial increase in risk immediately

following parturition with late first full-term pregnancy (see Figure 3.4.1), age-dependent changes in the magnitude and duration of parity-induced cellular and molecular alterations can markedly influence the overall breast cancer risk during the entire life span. Hence, in order to account for a significant life-long breast cancer protective effect, the early pregnancy-induced cellular and molecular alterations in mammary epithelial cell subtypes would have to dominate in magnitude and/or duration over late pregnancy-induced changes. Consequently, the scenarios 1,2,5,6 and 8 in Figure 6.2 have potentially the greatest biological significance. They imply either an equal magnitude but longer duration (scenarios 1,2), a greater magnitude and longer duration (scenarios 5,6) or a greater magnitude and similar duration (scenario 8) of early as compared to late pregnancy-induced cellular and molecular alterations in mammary epithelial cell subpopulations. In contrast, the scenarios 3,4 and 7 have comparatively little biological significance. To differentiate between the various possibilities, additional experiments are ongoing examining the magnitude and duration of altered expression of estrogen receptor, progesterone receptor, and the classic Wnt target versican in mammary glands after late pregnancy (i.e. age of mice at mating: 24 weeks instead of 6 weeks) and after a considerably longer post-weaning period (i.e. 78 weeks instead of 40 days), respectively. Notably, a recent study has already addressed the effect of late first full-term pregnancy on mammary gland characteristics in “postmenopausal” mice. This study found no changes in estrogen receptor expression and only minor decreases in progesterone receptor expression, indicating a reduced influence of late as compared to early pregnancy on mammary epithelial cell functionality (Raafat et al., 2012).

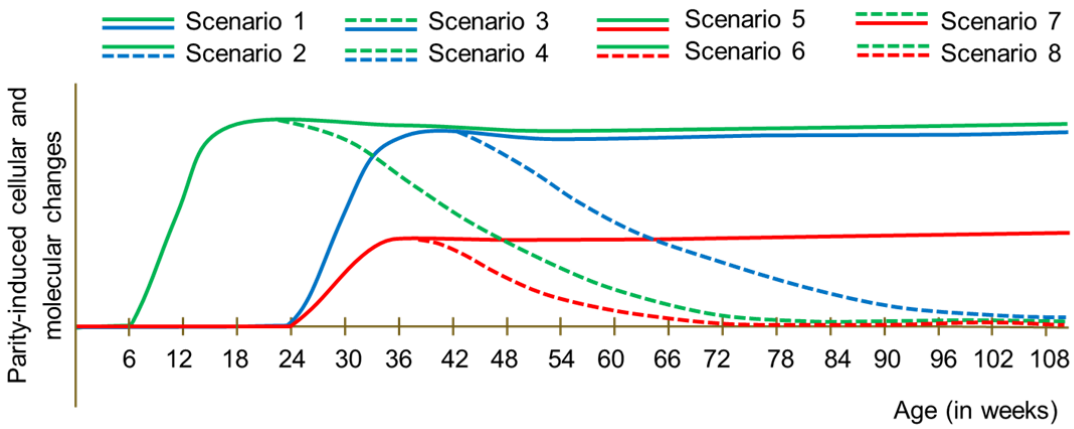

Figure 6.2 Magnitude and duration of parity-induced cellular and molecular changes in mammary epithelial cell subpopulations

After early and late first full-term pregnancy, eight scenarios are theoretically possible: 1) Parity-induced changes are of equal magnitude and of life-long duration after early and late pregnancy. 2) Parity-induced changes are of equal magnitude, and life-long after early but transient after late pregnancy. 3) Parity-induced changes are of equal magnitude, and transient after early but life-long after late pregnancy. 4) Parity-induced changes are of equal magnitude but of transient duration after early and late pregnancy. 5) Parity-induced changes are of smaller magnitude after late as compared to early pregnancy, but both are of life-long duration. 6) Parity-induced changes are of smaller magnitude after late as compared to early pregnancy, and life-long after early but transient after late pregnancy. 7) Parity-induced changes are of smaller magnitude after late as compared to early pregnancy, and transient after early but life-long after late pregnancy. 8) Parity-induced changes are of smaller magnitude after late as compared to early pregnancy, but both are of transient duration.

In conclusion, this study has identified major early parity-induced cellular and molecular alterations in mammary epithelial cell subtypes in mice. Specifically, downregulation of the Wnt/Notch signaling ratio and *in vitro* and *in vivo* proliferation defects were observed in basal mammary stem/progenitor cells. Mechanistically, the observed phenomenon can be explained by an early parity-induced reduction in hormone-sensitive and Wnt4-secreting luminal cells. Hence, the study provides direct experimental evidence for the hypothesis that early pregnancy changes the hormone responsiveness of the mammary gland and alters the properties and cell fates of specific mammary epithelial cell subpopulations. Furthermore, it opens the door for further studies investigating possibilities to mimic the breast cancer protective effect of early pregnancy.

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8. APPENDIX

Medina *Breast Cancer Research* 2013, 15:103
http://breast-cancer-research.com/content/15/3/103

EDITORIAL

Pregnancy protection of breast cancer: new insights reveal unanswered questions

Daniel Medina*

See related research by Meier-Abt *et al.*, http://breast-cancer-research.com/content/15/2/R36

Abstract

The recent paper by Meier-Abt and colleagues on pregnancy protection of breast cancer development takes a different approach to the problem and focused on the effect of parity on the cell subpopulations of the mouse mammary gland. Their results demonstrate that parity decreases the cell number of the hormone receptor-positive luminal cells (that is, luminal Sca1+) but not the basal stem/progenitor cells (CD24lo/CD49hi). Additionally, microarray studies demonstrate that wnt4 expression from the luminal Sca1+ cells is markedly reduced as is the wnt signaling pathway in basal cells. One important implication from these results is that targeting the wnt signaling pathway might be a feasible prevention approach in humans.

The recent paper by Meier-Abt and colleagues [1] on pregnancy protection of breast cancer development provides new insight into the mechanisms behind the protective effect. The decreased risk of breast cancer as a result of an early full-term pregnancy is a well-documented phenomenon in humans [2] and has been studied in multiple animal models [3]. The cellular and molecular changes resulting as a consequence of a full-term pregnancy are well documented in both animal models and humans [3-5]. Despite the abundant documentation of the effect of a full-term pregnancy over the past 40 years, the information has not been successfully translated to a clinical prevention paradigm.

Meier-Abt and colleagues [1] have taken a different approach to the problem and focused on the effect of parity on the cell subpopulations of the mouse mammary gland. The results are in many ways supportive of current concepts of pregnancy protection but have added the important knowledge of how pregnancy affects specific mammary cell subpopulations. They demonstrate quite convincingly that parity decreases the cell number of the hormone receptor-positive luminal cells (that is, luminal Sca1+) but not the basal stem/progenitor cells (CD24lo/CD49hi). Importantly, microarray studies demonstrate that wnt4 expression from the luminal Sca1+ cells is markedly reduced as is the wnt signaling pathway in the basal population. Additionally, Notch signaling in the basal cells is increased. In all cell populations, including the basal cells, differentiation markers are increased by parity. One important conclusion from their results is that the wnt signaling pathway is disrupted, which results in the division potential of the basal cells being severely impaired and, consequently, a predicted decrease in tumorigenic potential. Additionally, the repopulation potential of the basal cells from parous mice was shown to be decreased compared to the same population from age-matched virgin mice in an *in vivo* limiting dilution assay. One important implication from these results is that targeting the wnt signaling pathway might be a feasible prevention approach in humans. Of course, it will be important to demonstrate directly that this pathway has a causative role in a model of pregnancy-mediated protection.

Several important questions remain to be answered in our understanding of the cellular and molecular basis for parity-induced protection. First, the cell subpopulation that is the target for the initiation event that results in development of the cancer is not clear in any animal model and surely not in human. Thus, it is unknown if it is the basal stem cell or the alveolar progenitor cell that is the target for the oncogenic initiating event. Second, although the limiting dilution assay applied to subpopulations of the mammary epithelial cells is a sensitive assay to detect repopulation potential of cells, the assay itself has limitations. The assay has little relevance to the normal physiology of the mammary gland with respect to either turnover of the mammary cells during the estrus cycle or during pregnancy. The dynamics of mammary cell function, both differentiation activity and cell division activity, is a reflection of the cell-cell interactions.

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These include duct/alveoli cellular interactions as well as cell/microenvironment interactions [6]. The results of Meier-Abt and colleagues [1] illustrate this concept very nicely since decreases in cell number and specific gene expression are detected in the hormone receptor-positive cells and also the alteration of wnt signaling detected in the basal cells, which appears to be a consequence of the decreased wnt expression in the hormone receptor-positive cells. Since there is a clear cell-cell interaction in this pathway, what would be the result if one tested the repopulation ability of combined selected cell populations (that is, luminal (Sca1 positive) and basal cells)? It might reveal the repopulation frequency decrease from 1 in 500 cells to 1 in 100 cells as seen in several earlier experiments [7,8]. Additionally, with reporter labeled cells, one would be able to determine the cell that repopulates the gland under more normal physiological conditions. Recently, it has been demonstrated by lineage tracing that both the luminal cell and the basal cell generate the cells in their respective layers [9]. This is in accordance with the observations of others that, in pregnancy, the cell that generates the large expansion in cell number is the alveolar (lobular) progenitor cell, not the basal stem cell [10].

The role of the basal stem cell in normal mammary cell function and development is not well understood, particularly in the adult mammary gland. Although some studies suggest that pregnancy alters the stem cell population [11], other studies do not support this idea [12,13]. Indeed, using the serial transplantation assay to measure repopulation potential and replication life span, experiments have demonstrated there is no difference in these properties between stem cells taken from old multiparous and nulliparous mice [13]. A limiting dilution assay showed the same results as the serial transplantation assay [12,13]. The studies by Meier-Abt and colleagues [1] are important as they emphasize the critical interactions of the cell subpopulations and indicate that these interactions are important for proper interpretation of normal and neoplastic development in the mammary gland.

It is clear that as we understand more about the interactions of the different cell compartments of the mammary gland and the importance of the microenvironment in both the normal physiology of the gland and the tumorigenic process, we will have greater opportunities to target specific pathways. The results of Meier-Abt and colleagues [1] provide deeper understanding of the role of parity as well as identify new potential targets for prevention therapies.

Competing interests
The author declares that they have no competing interests.

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List of Abbreviations

ADAM17/TACE: tumor necrosis factor-α-converting enzyme

ADH: atypical ductal hyperplasia

Areg: amphiregulin

APC: adenomatous polyposis coli

Axin2: axis inhibition protein 2

BRCA1: breast cancer 1, early-onset

BRCA2: breast cancer 2, early-onset

CD24: cluster of differentiation 24 or heat stable antigen CD24

CD29: β1-integrin

CD49f: α6-integrin

DCIS: ductal carcinoma in situ

ECM: extracellular matrix

ER: estrogen receptor

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelial-to-mesenchymal transition

ErbB: erythroblastic leukemia viral oncogene homolog

FACS: fluorescence-activated cell sorting

FDR: false discovery rate

FEA: flat epithelial atypia

FGF: fibroblast growth factor

FGFR: fibroblast growth factor receptor

FWER: family-wise error rate

Fzd: Frizzled receptor

Gata3: GATA binding protein 3

GH: growth hormone

GSEA: gene set enrichment analysis

GSK-3β: glycogen synthase kinase beta

HER2 (in humans)/Neu (in rodents)/ErbB2: epidermal growth factor receptor 2

IDC: invasive ductal carcinoma

IGF: insulin-like growth factor

IGF-1R: insulin-like growth factor-1 receptor

Krt14: keratin 14

Krt18: keratin 18

LEF/TCF: lymphoid enhancer factor/T-cell-specific transcription factor

LN: lymph node

Lrp: LDL-receptor-related protein

MAML: mastermind-like

MARA: motif activity response analysis

MaSCs: mammary stem cells

MRUs: mammary repopulating units

NICD: Notch intracellular domain

N1ICD: intracellular domain of Notch1

N2ICD: intracellular domain of Notch2

N3ICD: intracellular domain of Notch3

N4ICD: intracellular domain of Notch4

p53: tumor protein 53

PI-MECs: parity-identified mammary epithelial cells

PR: progesterone receptor

PRL: prolactin

PrlR: prolactin receptor

PTEN: phosphatase and tensin homolog

RANKL: receptor activator for nuclear factor kappaB ligand

RBCs: red blood cells

RBP-J: recombination signal binding protein for immunoglobulin kappa J region

RTK: receptor tyrosine kinase

qPCR: quantitative PCR

Sca1: stem cell antigen1

Sfrp1: secreted frizzled-related protein1

SMA: smooth muscle actin

SMAD: mothers against DPP homolog

TDLU: terminal ductal lobuloalveolar unit

TEB: terminal end bud

TGFβ: transforming growth factor beta

TGFβR: transforming growth factor beta receptor

TNF: tumor necrosis factor

WBCs: white blood cells

Wnt: wingless related protein

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Publication list:

1) Meier-Abt F and M Bentires-Alj. Mechanisms underlying the breast cancer protective effect of an early pregnancy. – *In preparation.*

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Relevant scientific conferences attended, posters presented and talks given

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Annual ENBDC (European Network of Breast Development and Cancer Labs) workshop: Methods in mammary gland biology and breast cancer, April 24-26, 2009, and April 25-27, 2013, Weggis, CH (invited short talk given)

Gordon Research Conference for Mammary Gland Biology, June 10-15, 2012, Lucca, Italy (poster presented) and Mammary Gland Biology Gordon Research Seminar, June 9-10, 2012, Lucca, Italy (poster presented)

MD-PhD Retreats 2009-2012, CH (poster presented and invited short talk given)

FMI Annual Meetings 2009-2012, CH (posters presented)

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- Light microscopy, fluorescence microscopy
- Immunohistochemistry and immunofluorescent staining of organ sections, individual cells and cell colonies
- Mouse experimentation: generation of mouse models for studying possible mechanisms of early pregnancy-induced breast cancer protection
- Isolation of mammary epithelial cell subpopulations by fluorescence-activated cell sorting (FACS)
- *In vitro* colony formation assays and *in vivo* cell transplantation experiments
- Cell culture (of cell lines and of primary cells)
- Medical historical analyses with a focus on the process of drug approval
- Work with Xenopus oocytes as mRNA expression systems
- Organ perfusions, cell isolations, and transmembrane uptake experimentation in elasmobranches
- Phylogenetic analyses, protein structure predictions and protein modelling

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