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Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling

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Abstract

We previously demonstrated that Fibroblast Growth Factor 10 (FGF10) and its receptor FGFR2b play a key role in controlling the very early stages of mammary gland development during embryogenesis [Mailleux, A.A., Spencer-Dene, B., Dillon, C., Ndiaye, D., Savona-Baron, C., Itoh, N., Kato, S., Dickson, C., Thiery, J.P., and Bellusci, S. (2002). Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. Development 129, 53–60. Veltmaat, J. M., Relaix, F., Le, L.T., Kratochwil, K., Sala, F.G., van Veelen, W., Rice, R., Spencer-Dene, B., Mailleux, A.A., Rice, D.P., Thiery, J.P., and Bellusci, S. (2006). Gli3-mediated somitic Fgf10 expression gradients are required for the induction and patterning of mammary epithelium along the embryonic axes. Development 133, 2325–35.]. However, the role of FGFR2b signaling in postnatal mammary gland development is still elusive. We show that FGF10 is expressed at high level throughout the adipose tissue in the mammary gland of young virgin female mice whereas its main receptor FGFR2 is found mostly in the epithelium. Using a rtTA transactivator/tetracycline promoter approach allowing inducible and reversible attenuation of the FGFR2b signaling throughout the adult mouse, we are now reporting that FGFR2b signaling is also critical during postnatal mammary gland development. Ubiquitous attenuation of FGFR2b signaling in the postnatal mouse for 6 weeks starting immediately after birth is not lethal and leads to minor defects in the animal. Upon dissection of the mammary glands, a 40% reduction in size compared to the WT control is observed. Further examination shows a rudimentary mammary epithelial tree with completely absent terminal end buds (TEBs), compared to a well-branched structure observed in wild type. Transplantation of mammary gland explants into cleared fat pad of wild type mouse recipients indicates that the observed abnormal branching results from defective FGFR2b signaling in the epithelium. We also demonstrate that this rudimentary tree reforms TEBs and resumes branching upon removal of doxycycline suggesting that the regenerative capacities of the mammary epithelial progenitor cells were still functional despite long-term inactivation of the FGFR2b pathway. At the cellular level, upon FGFR2b attenuation, we show an increase in apoptosis associated with a decrease in the proliferation of the mammary luminal epithelium. We conclude that during puberty, there is a differential requirement for FGFR2b signaling in ductal vs. TEBs epithelium. FGFR2b signaling is crucial for the survival and proliferation of the mammary luminal epithelial cells, but does not affect the regenerative potential of the mammary epithelial progenitor cells.
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Keywords: *Fgf10*; *Fgfr2b*; Post-natal mammary gland development; Branching; Proliferation; Ductal epithelium; Terminal end buds

Introduction

Mammogenesis in mouse starts approximately on embryonic day 10.5 (E10.5) with the formation of two mammary lines running in an antero-posterior direction ventrally between fore and hind limbs, one line along each flank of the embryo

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(Veltmaat et al., 2003, 2004). At around E11.5, lens-shaped multilayered ectodermal structures (called placodes) can be detected slightly elevated above the surrounding ectoderm presumably along each mammary line at five reproducibly precise positions. These mammary placodes subsequently transform into bulbs of epithelial cells that are morphologically distinct from the surrounding epidermis. Approximately on E15.5, each bud elongates to form a sprout, invading the fat pad precursor. Each sprout forms a lumen, which opens on the surface of the skin, where the nipple forms concurrently by epidermal invagination. At about E16, the first ramifications of the sprouts occur, and by E18.5 the sprouts have developed into small, arborized glands. After birth, the gland grows isometrically with body growth. The postnatal mammary gland is mostly composed of ducts that contain two differentiated cell types, the luminal epithelial cells, which secrete milk protein, and the myoepithelial cells, which are located at the basal surface of the luminal cells. Before the start of puberty, the MG forms a rudimentary network of ductal epithelium. At the onset of puberty, at around 3 weeks of age, under the action of circulating hormones unique structures called terminal end buds (TEBs) form at the tips of the mammary ducts (for review see Veltmaat et al., 2003). These TEBs proliferate, ramify and actively invade the adipose tissue to allow the formation of a complex branching structure. This process takes place up to 10–12 weeks. After this developmental stage, the TEBs regress (Sternlicht et al., 2006). Little is known about the signaling pathways controlling in vivo the proliferation of the luminal cells, which are the cells mostly involved in breast cancer (Wiseman and Werb, 2002).

We previously reported the crucial role played by Fibroblast Growth Factor 10/Fibroblast Growth factor Receptor 2b (FGF10/FGFR2b) in the initial stages leading to the formation of the mammary placodes. The induction of most of the mammary line and four of the five mammary placodes on this line are under the control of FGF10 and FGFR2b (Mailleux et al., 2002; Veltmaat et al., 2006). We also reported that in *Fgfr2b* null embryos, the remaining mammary bud (mammary bud 4) regressed due to decreased proliferation and increased apoptosis in the mammary gland epithelium (Mailleux et al., 2002). This study therefore suggested that the FGFR2b pathway might be crucial for survival and proliferation of the mammary epithelial cells during postnatal development.

Indeed, an important role for FGFR2 signaling during postnatal mammary gland (MG) development is suggested by the fact that *Fgfr2* expression is maximal in mature virgin mice, declines during pregnancy and lactation, but increases after weaning (Pedchenko and Imagawa, 2000). The rise in *Fgfr2* mRNA in the virgin animal corresponds to a significant increase in the branching of the mammary epithelial tree. During ductal development, the genes encoding the two main FGFR2b ligands, FGF10 and FGF7, are expressed at a ratio of 15 to 1 respectively (Pedchenko and Imagawa, 2000). The expression of *Fgfr2* and its associated ligands in the MG during ductal development suggests an important role for the FGFR2b pathway in post-natal growth of the mammary epithelium. Such a positive role for FGFR2b signaling in epithelial growth is re-enforced by the fact that FGF10, which mostly binds to FGFR2b, has been described as an oncogene in breast cancer cells (Theodorou et al., 2004). In addition, *FGFR2* is amplified and overexpressed in breast cancer (Grose and Dickson, 2005; Moffa and Ethier, 2007). Mutations in *FGFR2* have recently been strongly associated with a higher risk of breast cancer in postmenopausal women with no previous family history of breast cancer (Hunter et al., 2007).

The role of FGF10/FGFR2b signaling in the epithelial/mesenchymal interactions that characterize postnatal MG development is demonstrated through the analysis of the MG phenotype of transgenic mice allowing inducible and reversible attenuation of the FGFR2b pathway throughout the whole adult mouse upon addition of doxycycline. This study demonstrates that FGFR2b signaling in mammogenesis is not only critical during embryogenesis but also during postnatal development.

Materials and methods

Analysis of LacZ expression

Fgf10/LacZ expression was monitored in $Fgf10^{LacZ}$ mice (Kelly et al., 2001) by β-galactosidase activity using whole-mount and histological revelation as described by Kelly et al. (1995). Mammary glands (MGs) from 3-week-old virgin females were fixed 2 h in 4% PFA. Following the fixation, the MGs were washed in 1× PBS and stained in X-gal solution. A similar protocol was carried out for MG from *Rosa26* mice (Zambrowicz et al., 1997).

Generation of rtTA; tet(O)sFgfr2b animals

CMV-Cre mice (Schwenk et al., 1995) were crossed with $rtTA^{flox}$ mice (Belteki et al., 2005) to generate *rtTA* mice expressing rtTA from the *Rosa26* promoter in every single cell of the body. *rtTA* mice are now crossed with *tet(O) soluble Fgfr2b* mice (Hokuto et al., 2003) to generate double transgenic *rtTA; tet (O)soluble Fgfr2b* mice. These mice are on the CD1 mixed genetic background and allow inducible and reversible attenuation of the FGFR2b pathway in the embryo or mouse simply by feeding the mice with doxycycline containing food (Rodent diet with 0.0625% Doxycycline, Harlan Teklad TD01306). The reversibility of the phenotype is studied by putting the treated mice on normal food. Genotyping of each allele was done as previously described (Schwenk et al., 1995; Belteki et al., 2005; Hokuto et al., 2003).

Antibodies

MG from 3-week-old female mice were fixed in 4% PFA overnight and stored in 70% ethanol. The tissues were embedded in paraffin and 5 μm longitudinal sections were made. IHC was performed with the Envision kit from Dako cytomation. FGFR1 antibody (Flg, 1:200, Santa Cruz Inc.) and FGFR2 (Bek, 1:200, Santa Cruz, Inc) were incubated overnight at 4 °C as previously described (Sala et al., 2006). MG paraffin sections were treated with Cy3 conjugated-monoclonal anti α-SMA Ab (1:200, Sigma, clone 1A4) as previously described (De Langhe et al., 2005). Photomicrographs were taken using a Leica DMRA fluorescence microscope with a Hamamatsu Digital CCD Camera.

Proliferation

One of the two MGs number 4 from P35 WT and double transgenic animals ($n=3$ for each) non-induced with dox (control group) were surgically removed and the operated mice were put on doxycycline food for 1 week to induce gene expression. Similar experiments were also carried out with P60 double transgenic animals ($n=3$) to assess the role of FGFR2b signaling in late puberty. After a week, the animals were sacrificed and the second mammary glands 4 were removed (experimental group). The mammary glands were fixed