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Recommended Citation
Elo, Teresa; Lindfors, Päivi H.; Lan, Qiang; Voutilainen, Maria; Trela, Ewelina; Ohlsson, Claes; Huh, Sung-Ho; Ornitz, David M.; Poutanen, Matti; Howard, Beatrice A.; and Mikkola, Marja L., "Ectodysplasin target gene Fgf20 regulates mammary bud growth and ductal invasion and branching during puberty." Scientific Reports. 7, (2017). https://digitalcommons.wustl.edu/open_access_pubs/6000

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Ectodysplasin target gene Fgf20 regulates mammary bud growth and ductal invasion and branching during puberty

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Mammary gland development begins with the appearance of epithelial placodes that invaginate, sprout, and branch to form small arborized trees by birth. The second phase of ductal growth and branching is driven by the highly invasive structures called terminal end buds (TEBs) that form at ductal tips at the onset of puberty. Ectodysplasin (Eda), a tumor necrosis factor-like ligand, is essential for the development of skin appendages including the breast. In mice, Eda regulates mammary placode formation and branching morphogenesis, but the underlying molecular mechanisms are poorly understood. Fibroblast growth factor (Fgf) receptors have a recognized role in mammary ductal development and stem cell maintenance, but the ligands involved are ill-defined. Here we report that Fgf20 is expressed in embryonic mammary glands and is regulated by the Eda pathway. Fgf20 deficiency does not impede mammary gland induction, but compromises mammary bud growth, as well as TEB formation, ductal outgrowth and branching during puberty. We further show that loss of Fgf20 delays formation of Eda-induced supernumerary mammary buds and normalizes the embryonic and postnatal hyperbranching phenotype of Eda overexpressing mice. These findings identify a hitherto unknown function for Fgf20 in mammary budding and branching morphogenesis.

Mammalian fibroblast growth factors (Fgfs) constitute a family of 18 secreted polypeptide growth factors with diverse roles in multiple developmental processes. Secreced Fgfs serve as ligands for single-pass transmembrane receptor tyrosine kinases (Fgfr1–4). With the exception of Fgfr4, alternative splicing of Fgfrs produces two isoforms (IIIb and IIIc isoforms) with different ligand binding specificities. In general, mesenchymal Fgfs engage epithelial IIIb receptor isoforms, whereas epithelial Fgfs signal to mesenchymal IIIc receptors. Activation of any of these isoforms can trigger several signalling cascades including the RAS-MAPK, PI3K, STAT, and PLCγ pathways leading to various cellular responses in a context dependent manner. The fact that all Fgfs can bind to several Fgfrs, and vice versa, produces a high degree of redundancy within the system.

Mammary gland development proceeds via distinct stages: the hormone-independent embryonic and prepupal stages, and the subsequent pubertal, pregnancy, lactation, and involution stages driven by hormonal cues. In mice, mammary gland development commences at embryonic day 11 (E11) with the sequential appearance of five pairs of mammary primordia called placodes. Placodes are local epithelial thickenings that gradually invaginate to the underlying tissue to form buds, which from E12.5 onwards are surrounded by a specialized condensed mammary mesenchyme. Mammary buds grow relatively slowly in size until E15–E16 when a primary sprout forms. The sprout invades the secondary mammary mesenchyme, the precursor of the fatty adult...
Mammary gland morphogenesis relies on sequential and reciprocal crosstalk between the epithelium and the underlying stroma and these tissue interactions guide all aspects of mammary gland development from induction to involution. This crosstalk is mediated by conserved signaling pathways, of which the Wnt and Fgf pathways are the most critical ones during the early stages of mammogenesis. Loss of the Wnt signal mediator Lef1 leads to absence of placodes and, whereas intestinal overexpression of the soluble Wnt inhibitor Dkk1 prevents mammary placode formation altogether. Deficiency in Fgf10 or its receptor Fgfr2b blocks induction of all mammary placodes except the fourth. The initiation of bud outgrowth is triggered by epithelially expressed parathyroid hormone related peptide (PTHrP), a.k.a. parathyroid hormone like hormone: mice null for PTHrP or its mesenchymal receptor (Pthlr) display little to no bud sprouting. Disruption of canonical Wnt signaling pathway components, such as Lrp6 results in stunted embryonic branching morphogenesis and underdeveloped fat pad. Ductal growth and branching is also compromised, albeit to a lesser extent, in epidermal growth factor receptor (Egfr) null neonates.

Pubertal branching morphogenesis is regulated by systemic hormones, especially estradiol and growth hormone (GH). A critical factor induced by estrogen receptor α (Esr1) in the mammary epithelium is the Egfr family member amphieregulin (Areg), which activates stromal Egfr signaling. Accordingly, Areg and Esr1 knockout mice display a highly similar pubertal phenotype characterized by failure in TEB formation and ductal elongation. GH signalling is essential in the mammary stroma where its effects are mediated by insulin-like growth factor 1 (Igf-1), which in turn promotes epithelial cell proliferation and survival. Both GH receptor and Igf-1 null mice exhibit greatly limited ductal outgrowth. In addition, several other signaling molecules regulate ductal morphogenesis during puberty although their link to hormone signaling is less clear. Also Fgf signaling plays an important role in mammary branching morphogenesis, as well as in maintaining homeostasis in the adult. Thus far, functional studies have focused on epithelial Fgfrs, in particular Fgfr2b, or their stromal ligands. The single pair (bud 4) of Fgf10 null mammary primordia sprouts, but shows either absent or very rudimentary ramifications. Several studies have demonstrated the crucial role of Fgfr2 signaling in postnatal development including induction and maintenance of the TEBs, and in ability of mammary stem cells to repopulate the fat pad in transplantation assays. Furthermore, conditional epithelial deletion of Fgfr1 leads to a ductal outgrowth phenotype, albeit transient, which is evident already at the onset of puberty.

Ectodysplasin-A1 (hereafter Eda), a member of the tumor necrosis factor (Tnf) superfamily has recently emerged as an important regulator of mammary gland development. Eda signaling is mediated via its receptor Edar and culminates in the activation of the transcription factor NF-κB. Eda pathway loss- and gain-of-function mouse models have been highly informative in elucidating the role of this pathway in mammary gland biology. Eda is dispensable for mammary placode formation, yet Eda-overexpressing (K14-Eda) mice develop supernumerary mammary glands along and anterior to the milk line. Edar, or NF-κB leads to smaller ductal trees, a phenotype that manifests from embryogenesis up to at least 6 weeks of age. The converse is observed in Eda and Edar overexpressing mice. In humans, inactivating mutations in the Eda pathway genes cause a syndrome characterized by defective development of several ectodermal organs including the breast. In order to identify the transcriptional mediators of Eda/Edar/NF-κB, we performed microarray profiling of embryonic Eda null mammary buds after a 4-hour ex vivo exposure to control medium or recombinant Eda protein. This screen revealed several putative Eda target genes including Fgfr2, one of the Fgfr family members reported to be expressed in the mammary buds, and Fgf17. Two Fgfr family members reported to be expressed in the mammary buds, but not upregulated by Eda in the microarray. In line with the microarray results, after 4 hours Eda-treatment, of these only Fgfr2 and Fgf17 were upregulated 5.8-fold (p = 0.042) and 3.8-fold (p = 0.019), respectively (Fig. 1a). Analysis of the absolute miRNA quantity indicated that Fgfr2 is expressed at a very low level, and thus the role of Fgfr2 in mammogenesis was not analyzed further.

In order to analyze expression of Fgf20 in embryonic mammary glands, we took advantage of the Fgf20-LacZ knock-in allele and performed X-gal staining on Fgf20−/−/embryos at E10.5 and E18.5. Expression of Fgf20-LacZ was detected earliest at ~E11.25 in the mammary bud (data not shown), and at E11.5 in the buds 1 and 3 (Fig. 1b). At E13.5, Fgf20-LacZ expression was detected in all mammary buds (Fig. 1b) and accordingly, in situ hybridization with an Fgf20 specific probe showed positive signal in wildtype embryos at the same stage (Fig. 1d). The Fgf20-LacZ expression was still relatively strong in the mammary buds at E15.5 (Fig. 1e) but was substantially downregulated at E16.5 (Fig. 1f). At E18.5, no expression of Fgf20-LacZ could be detected in the mammary glands by X-gal staining (Fig. 1h) or immunohistochemical staining with anti-β-galactosidase antibody, although expression in hair follicles was readily observed (Fig. S1a), as reported previously. At postnatal
stages, expression of Fgf20-LacZ was assessed by X-gal staining and anti-β-galactosidase antibody in mammary glands of 3-, 5- and 7-week-old Fgf20 LacZ/+ and Fgf20LacZ/LacZ mice, and by qRT-PCR in samples from 3 different regions (proximal to nipple, middle, and distal to nipple) of 5-week old glands. No expression was detected in the postnatal mammary gland by any of the methods used (Supplementary Fig. S1).

Eda levels influence the expression of Fgf20 in vivo. The observation that Eda induced the expression of Fgf20 in the embryonic mammary buds ex vivo prompted us to study the influence of Eda on Fgf20 expression levels in vivo by analyzing the Fgf20-LacZ expression in Eda null and Eda-overexpressing (K14-Eda) embryos. In Eda−/− embryos there was a slight delay in the onset of Fgf20-LacZ expression at E11.5 followed by somewhat decreased signal at E12.5 compared to control or K14-Eda embryos (Fig. 2a,b). At E13.5–E14.5 expression in K14-Eda embryos appeared more intense (Fig. 2c,d), and at E15.5, Fgf20-LacZ expression levels correlated with the Eda status (Fig. 2e). Together, these data show that loss- and gain- of Eda influence Fgf20-LacZ expression.

Figure 1. Fgf20 is induced by Eda and is expressed in embryonic mammary glands. (a) qRT-PCR analysis of Fgf4 (n = 4), Fgf9 (n = 4), Fgf17 (n = 6) and Fgf20 (n = 7) expression in E13.5 Eda−/− mammary buds after 4 h treatment with Eda protein ex vivo. Values represent mean ± SD. (b,c) X-gal-stained whole mounts of Fgf20lacZ/+ embryos at E11.5 (b) and E13.5 (c) showing positive staining in the developing mammary buds (numbered). (d) In situ hybridization of a WT embryo with an Fgf20 specific probe at E13.5. (e,f) X-Gal stained whole mount of E15.5 whole embryo (e) and dissected skin of E16.5 embryo (f) showing staining in the developing mammary buds (numbered) and hair follicles. (g,h) Representative figures of histological sections of X-Gal whole mount-stained mammary glands of Fgf20lacZ/+ embryos at E16.5 (g) and E18.5 (h). *p < 0.05. At least two litters of Fgf20lacZ/+ embryos per stage were analyzed. *p < 0.05. mb, mammary bud.
although modestly, yet clearly cues other than Eda have a more prominent impact on Fgf20 expression during embryogenesis. The Wnt pathway is the most likely positive regulator: the murine Fgf20 promoter is known to be highly responsive to β-catenin/Lef1 in promoter-reporter assays.

Absence of Fgf20 compromises mammary bud formation. To elucidate the role of Fgf20 in mammary gland development, we first analyzed the expression of placode markers Wnt10b and PTHrP by RNA in situ hybridization in the mammary buds of Fgf20LacZ/+ and Fgf20LacZ/LacZ mice (Figs 3 and 4). At 46–48 somite stage (E11.5–E11.75) Wnt10b expression in the two genotypes was indistinguishable indicating that Fgf20 deficiency does not impede induction of mammary gland development (Fig. 3a). At E12.5, however, Wnt10b expression domain appeared smaller in Fgf20LacZ/LacZ embryos, the difference being most pronounced in bud 3 (Fig. 3b), which is the first bud to form. Quantification of the Wnt10b expression domain confirmed a significant difference between the two genotypes (p = 0.0007) (Fig. 3b'). At E13.5, the same was observed with the PTHrP probe, or when Fgf20-LacZ expression was assessed by X-gal staining (Fig. 4). For a more detailed morphological analysis, EpCAM-stained mammary buds 3 were visualized by whole mount confocal microscopy in 3D (Fig. 3c,d).

The appearance of supernumerary mammary placodes between the endogenous buds 3 and 4 in K14-Eda mice has been shown at E12.5 by a number of Wnt pathway genes, and at E13.5 they are clearly visible by various
mammary bud markers including PTHrP\textsuperscript{29,33,34}. At E13.5 PTHrP was not detected between bud 3 and 4 in K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} embryos in contrast to K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} embryos (Fig. 4a). Furthermore, stereomicroscopic inspection, as well as X-gal staining and subsequent analysis of histological sections suggested the absence of supernumerary mammary buds in the K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} mice at E13.5 (Fig. 4b). However, based on similar analyses, supernumerary mammary buds were detectable in K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} embryos slightly later, at ~E14.0 (Supplementary Fig. S3). Accordingly, supernumerary nipples were observed on the milk line and the neck region of pre- and post-pubertal K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} females (Supplementary Fig. S3). As previously reported for K14-Eda males\textsuperscript{34}, also K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/+} and K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} males had supernumerary nipples present.

**Figure 3.** Fgf20 deficiency does not impede placode induction but compromises bud growth. (a) Expression of Wnt10b at somite stage 46–48 (Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 7; Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 6), and (b) quantification of Wnt10b expression area (mammary bud 3) at E12.5. (d,e) 3D images and volume quantifications of EpCAM-stained mammary bud 3 at E13.5 (Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 24; Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 28), and E15.5 (Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 8; Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 13). The bud contours were outlined manually (purple) for volume quantification. ***p < 0.001; ****p < 0.0001.

**Figure 4.** Fgf20 deficiency delays induction of supernumerary buds in K14-Eda mice. (a) Expression of PTHrP at E13.5 (Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 4; Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 4; K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 7; K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 5), and (b) X-gal staining of Fgf20-LacZ at E13.5 (Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 4; Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 6; K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/+}, n = 11; K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}}, n = 8). Supernumerary placodes (stars) were detected between buds 3 and 4 in K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/+} embryos at E13.5, but not in K14-Eda;Fgf20\textsuperscript{a\textsubscript{Zl}/a\textsubscript{Zl}} embryos.
nipples, and at least in the neck region, a ductal tree was readily observed in compound mutants (Supplementary Fig. S3). In conclusion, in the absence of Fgf20, all mammary buds formed, yet a clear reduction in bud size and a slight delay in appearance of supernumerary mammary buds in K14-Eda embryos was evident.

**Absence of Fgf20 delays ductal growth in puberty.** Macroscopic analysis of pubertal and adult Fgf20LacZ/LacZ females revealed the presence of the normal number of nipples. To examine the impact of Fgf20 deficiency on postnatal mammary morphogenesis, 4th mammary glands of 5-week-old WT and Fgf20LacZ/LacZ were analyzed (Fig. 5a–c). The number of the ductal ends was reduced by 35% (p = 0.0038) in Fgf20LacZ/LacZ mice compared to WT controls (Fig. 5d,e). Also, the extent of ductal outgrowth (i.e. penetration to the fat pad) was significantly compromised (p = 0.037) (Fig. 5f). These data clearly show that absence of Fgf20 greatly retards ductal outgrowth during puberty. The ductal characteristics were, however, quite variable among the Fgf20LacZ/LacZ mice: often the ductal tree was very rudimentary and barely contained any TEBs while in some mice the ductal tree was only modestly affected (Fig. 5a–c). Quantification of the maximum width of the five largest TEBs/ductal tips in each specimen confirmed a significant difference between Fgf20LacZ/LacZ and WT mice (p = 0.029) (Fig. 5g). Ki-67 expression analysis in TEBs evidenced a decrease in the number of proliferating cells in Fgf20 mutants (p = 0.0038) (Fig. 5h,i).

**No evidence for a systemic pubertal defect in Fgf20LacZ/LacZ females.** We detected Fgf20 expression only in the embryonic mammary glands (as above), yet Fgf20LacZ/LacZ mammary glands displayed a remarkable postnatal phenotype (Figs 5 and 6). To assess whether the pubertal phenotype could be caused by a systemic defect due to the germline deletion of the Fgf20 gene, we analyzed various parameters in the mutant animals. We found no difference in the size of the animals at the onset of, or during puberty (at 3, 5, or 7 weeks of age), or the weight of ovaries and uteri (Supplementary Fig. S4). Yet, 18% of 7-week-old Fgf20LacZ/LacZ females (n = 22) had completely closed vaginas, whereas a similar defect was not observed in WT mice (n = 9). These mice were not used for mammary gland analyses. The estrus cycles analyzed from vaginal smear cytology of WT and Fgf20LacZ/LacZ females were normal, and serum estradiol levels of the 7-week-old Fgf20LacZ/LacZ females in diestrus were similar to those of WT littermates (Supplementary Fig. S4). Finally, we performed mammary fat pad transplantations in which 1 mm^3 pieces of adult WT epithelium were transplanted into the cleared fat pad of 3-to-4-week old WT or Fgf20LacZ/LacZ females and allowed to grow for 5 weeks before analysis. WT epithelium grew equally well in the fat pad of both recipients (Supplementary Fig. S4). Collectively, these data indicate that there is no gross systemic defect in Fgf20LacZ/LacZ females, which could explain the pubertal mammary phenotype.

**Absence of Fgf20 normalizes the hyperbranching phenotype of K14-Eda mice.** Our data showing that Fgf20 expression levels are modulated by Eda (Fig. 1) and loss of Fgf20 delays ductal growth at puberty (Fig. 5) prompted us to study the effects of Fgf20 deficiency on ductal branching at other developmental stages, as well as the crosstalk with the Eda pathway. At E18, the number of ductal ends in the mammary glands of Fgf20LacZ/LacZ embryos was similar to that of wildtype mice (p = 0.638) (Fig. 6a,a'). However, mammary glands of 3-week-old Fgf20LacZ/LacZ mice contained somewhat lower number of ductal tips than those of WT controls (p = 0.0321) (Fig. 6b,b'). At 7 weeks of age, the decrease in the ductal outgrowth and number of ductal ends in Fgf20LacZ/LacZ mice was prominent (p = 0.0039 and p = 0.0051, respectively) (Fig. 6c,c',e), even more pronounced than at 5 weeks of age (Fig. 5d). However, at 12 weeks of age, the number of ductal ends was similar in both genotypes (p = 0.363) (Fig. 6d,d').

Consistent with our previous results 34, the number of ductal ends was significantly higher in K14-Eda mice compared to WT controls at E18 (p = 0.00009) and 3 weeks of age (p = 0.0019) (Fig. 6a,a,b,b'). The hyperbranching phenotype was apparent also at 7 (p = 0.034) and 12 weeks of age (p = 0.0004) (Fig. 6c,c,d,d'). Surprisingly, even though Fgf20 null mammary glands did not display a growth phenotype at E18, the K14-Eda phenotype was greatly attenuated in Fgf20LacZ/LacZ background (p = 0.0005) (Fig. 6a). Also at later stages, loss of Fgf20 normalized the K14-Eda phenotype, although at 7 weeks of age, the difference did not reach statistical significance (p_{int} = 0.0046; p_{adj} = 0.1521, p_{2w} = 0.0011). These data identify Fgf20 as a critical mediator of Eda in mammary ductal growth and branching.

**At late puberty, the terminal end buds of Fgf20LacZ/LacZ mice are larger and more proliferative.** Since the growth delay of the Fgf20 mutants was most pronounced at 7 weeks of age, we analyzed the ducts and TEBs of Fgf20LacZ/LacZ and WT glands in more detail at this stage. The architecture of the ducts appeared normal based on all criteria used: histology, hormone receptor expression, the distribution of basal (K14) and luminal (K8) keratins, and the expression of basal cell marker α-SMA (Supplementary Fig. S5). Accordingly, FACS analysis did not show significant differences in the percentage of luminal (CD29^-/CD24^+) or basal (CD29^-/CD24^+) cells between WT and the Fgf20LacZ/LacZ mice at 7 week of age, nor at 3 weeks when the growth phenotype was first evident (Supplementary Fig. S5).

Analysis of TEBs, however, revealed that the epithelium appeared more cellular in Fgf20LacZ/LacZ mice compared to WT mice (Fig. 7a). TEB area, measured from the carmine alum whole mount images, was larger in Fgf20LacZ/LacZ mice at the same age (Fig. 7b). Quantification of Ki-67 and cleaved caspase-3 positive cells in TEBs revealed that the proportion of the proliferating cells was significantly higher in Fgf20LacZ/LacZ mice compared to WT controls (Fig. 7c,c'), but there was no difference in the proportion of apoptotic cells (Fig. 7d,d'). ERα and PR expression was indistinguishable between WT and Fgf20LacZ/LacZ TEBs (Fig. 7e,f). TEBs consist of a mass of luminal K8+ body cells surrounded by α-SMA+ /p63+ cap cell layer. The expression patterns of body and cap cell markers were unchanged in 7-week old Fgf20LacZ/LacZ mice (Fig. 7g–i) indicating intact TEB architecture and cell identities.
Discussion

In the current study, we have unveiled a role for Fgf20 in two stages of embryonic mammary gland development: budding and branching morphogenesis. Even though Fgf20 was dispensable for mammary placode induction, the buds were smaller in size. The molecular mechanism underlying the bud growth defect remains elusive. Furthermore, loss of Fgf20 delayed, but did not prevent, the formation of supernumerary mammary buds in K14-Eda embryos. Perinatally, Fgf20 null mammary glands did not differ from the WT controls, yet the K14-Eda hyperbranching phenotype was greatly attenuated in Fgf20 null background. The most plausible
explanation for these seemingly contradictory findings is redundancy of Fgf20 with other Fgf ligands, the most prominent candidate being Fgf9, a member of the same Fgf subfamily. Fgf9 is expressed in embryonic mammary glands, shares similar biochemical properties with Fgf20 including receptor specificity, and redundancy between these two Fgfs has already been demonstrated in developing teeth, kidney, and cochlea. Other Fgfs reported to be expressed in mammary bud epithelium are Fgf4, Fgf8, and Fgf17, which may further compensate for loss of Fgf20.

Fgf signaling typically mediates crosstalk across tissue compartments, but whether the effects of Fgf20 on mammary gland epithelium are direct, mediated by the stroma, or both, is currently unknown. Fgf20 preferentially, but not exclusively, activates the mesenchymally expressed IIIc receptors isoforms. In the developing

Figure 6. Loss of Fgf20 attenuates the K14-Eda hyperbranching phenotype. (a–d) Carmine alum stained 4th mammary gland of WT, Fgf20/+/LacZ, K14-Eda, and K14-Eda;Fgf20/+/LacZ mice at E18 (a), 3 weeks (b), 7 weeks (c), and 12 weeks of age (d). (a’–d’) Quantification of the total number of end ducts (a’b’) or end ducts past the lymph node (c’d’) in 4th mammary gland. Number of glands analyzed were: WT (nE18 = 5, n3wk = 18, n7wk = 12, n12wk = 15) Fgf20/+/LacZ (nE18 = 8, n3wk = 16, n7wk = 28, n12wk = 5), K14-Eda (nE18 = 7, n3wk = 8, n7wk = 9, n12wk = 13) and K14-Eda;Fgf20/+/LacZ (nE18 = 6, n3wk = 8, n7wk = 9, n12wk = 10) (e) Ductal outgrowth (mm) measured from center of the lymph node in Fgf20/+/ (nGlands = 7) and Fgf20/+/LacZ (nGlands = 23). Data are shown as mean ± SD. ***p < 0.001; **p < 0.01; *p < 0.05; NS, not significant.
cochlea, epithelially expressed Fgf20 positively regulates epithelial progenitor proliferation via the mesenchyme, whereas intraepithelial Fgf20 signaling is essential for sensory cell differentiation. In hair follicles, Fgf20 is dispensable for placode formation, but is necessary for condensation of the underlying mesenchymal cells, which in turn is required for further follicular downgrowth. The target genes regulated by Fgf20 have remained elusive in all organs studied so far.

We have previously shown that Eda regulates expression of Fgf20 in embryonic hair follicles and teeth where Fgf20 functions as one of the major downstream effectors of the Eda pathway. Here, we identify Fgf20 as a mediator of Eda in the developing mammary glands: absence of Fgf20 delayed formation of supernumerary buds and normalized the hyperbranching mammary phenotype of K14-Eda mice, an effect maintained until adulthood. However, our data implicate the existence of other downstream targets of Eda besides Fgf20, since at E18 and at the onset of puberty, the ductal trees of Eda-null mice are more severely affected than those of Fgf20LacZ/LacZ mice. Our earlier studies have identified several other Eda-induced factors that can enhance branching morphogenesis such as PTHrP, Egfr ligands, and Wnt pathway agonists. Hence, the Eda-null and K14-Eda branching phenotypes are likely the combinatorial result of changes in the expression level of multiple Eda target genes.

Our data show that Fgf20 has a considerable impact on postnatal mammary morphogenesis since its absence led to defective TEB formation and delayed ductal invasion during puberty. However, the ductal growth defect was transient: the ductal trees caught up to the WT glands between 7 and 12 weeks of age. We propose that this also explains the counterintuitive finding of increased cell proliferation in Fgf20LacZ/LacZ TEBs at 7 weeks of age. In WT glands, the percentage of proliferative cells in the TEBs decreases between 3 weeks of age and late puberty, whereas Fgf20LacZ/LacZ mammary glands begin their growth burst at 7 weeks of age.

The embryonic phenotype and the subtle reduction in the number of branches in 3 weeks old Fgf20LacZ/LacZ mice implicates that the defect underlying the pubertal ductal phenotype may arise before puberty. We were unable to detect Fgf20 expression during puberty, not even by qRT-PCR, a finding in line with a recent study assessing Fgf20 expression in mammary glands of 3, 5, and 10 week old mice. Thus, it is plausible that Fgf20 deficiency during embryogenesis leads to qualitative changes in the mammary stem/progenitor cells that fully manifest only during puberty. Fittingly, a recent study implicated epithelial Fgfr1/2 signaling in proper mammary stem cell function during development. However, we cannot exclude the possibility that Fgf20 is expressed during puberty in a rare cell population that escaped our analysis. To answer the question whether Fgf20 has a role in pubertal development independent of its embryonic function must await for the generation of a conditional Fgf20 mouse.

The mammary phenotype of Fgf20LacZ/LacZ mice resembles the phenotypes generated by K14-Cre-mediated deletion of Fgfr1 and MMTV-Cre-mediated (mosaic) deletion of Fgfr2, which both display compromised TEB

**Figure 7.** Analysis of terminal end buds of Fgf20LacZ/LacZ mice at 7 weeks of age. (a) Hematoxylin Eosin-stained sections of WT and Fgf20LacZ/LacZ TEBs. (b) Quantification of TEB area from Carmine alum stained mammary glands of WT (n = 9) and Fgf20LacZ/LacZ (n = 12) mice. (c,c') Immunohistochemical staining and quantification of Ki-67-positive cells in TEBs of WT (n = 4) and Fgf20LacZ/LacZ (n = 5) mice. Total number of TEBs analyzed was n = 26 (WT), n = 30 (Fgf20LacZ/LacZ). (d,d') Immunohistochemical staining and quantification of cleaved caspase-3-positive cells in WT (n = 4) and Fgf20LacZ/LacZ mice (n = 4). Total number of TEBs analyzed was n = 34 (WT), n = 29 (Fgf20LacZ/LacZ). (e-i) Immunohistochemical staining of ERα (e), PR (f), K8 and K14 (g), SMAα (h), and p63 (i) in the TEBs of WT and Fgf20LacZ/LacZ mice. Minimum of 4 mice per genotype were analyzed. Values represent mean ± SD. *p < 0.01; *p < 0.05; NS, not significant.
formation, reduced number of branch points, and pubertal ductal outgrowth defect that normalizes in the adulthood. A complete failure in TEB maintenance is observed in mice inducibly overexpressing a transgene encoding a soluble form of Fgfr2b. Interestingly, upon cessation of transgene expression 6 weeks after its induction, TEBs reform and branching is resumed. These data are suggestive of Fgfr signaling being essential for the functionality rather than survival of mammary stem/progenitor cells driving TEB formation and ductal invasion.

Pubertal ductal morphogenesis is a complex hormone regulated process, which involves cellular functions such as proliferation, apoptosis, migration, ECM degradation, and a tight interplay between epithelial and mesenchymal compartments. A great number of genetically manipulated mice, and experiments using slowly-releasing protein pellets, are known to cause a pubertal mammary phenotype. These studies show that loss of tissue integrity in TEBs readily leads to ductal outgrowth defects. However, this is unlikely the case in Fgf20-/-/LacZ mice, as the expression pattern of body and cap cell markers was unaltered. Another important class of pubertal phenotypes is caused by loss- or gain-of-function of matrix remodeling enzymes such as matrix metalloproteinases (MMPs), which regulate ductal invasion and branching via their ability to sculpt the ECM, and Fgfr1/2 stimulation has been shown to induce the expression of Mmp3 and Mmp9 in several breast cancer and immortalized mammary epithelial cell lines.

In conclusion, our results identify a hitherto unknown function for Fgf20 in both embryonic and postnatal mammary gland morphogenesis. Our data suggest that compromised Fgf20 signaling during embryogenesis results in qualitative changes in TEBs that are thought to harbor the majority of stem cells driving branching morphogenesis during puberty. To our knowledge, in addition to Fgf10, Fgf20 is the only Fgf family member with a proven in vivo function in mammary gland development. Furthermore, we discovered Fgf20 as an important mediator of Eda in mammary gland budding and branching morphogenesis. Future studies should shed light on the molecular mechanisms downstream of Fgf20 in mammary gland morphogenesis.

Materials and Methods

Mice. The generation and genotyping of Fgf20-/-/LacZ, K14-Eda, and Eda-/- (Tabby; Jackson Laboratories, stock no 000314) mouse strains have been described previously. Fgf20-/-/LacZ and K14-Eda mice were maintained in the C57Bl/6 background (K14-Eda > 10 generations and Fgf20-/-/LacZ > 5 generations) and Eda-/- mice in the B6CBA background. Embryonic ages were defined based on the appearance of vaginal plug and external hind. A complete failure in TEB maintenance is observed in mice inducibly overexpressing a transgene encoding a soluble form of Fgfr2b. Interestingly, upon cessation of transgene expression 6 weeks after its induction, TEBs reform and branching is resumed. These data are suggestive of Fgfr signaling being essential for the functionality rather than survival of mammary stem/progenitor cells driving TEB formation and ductal invasion.

Embryonic mammary bud cultures and quantitative RT-PCR. The hanging drop culture method used for the embryonic mammary bud cultures has been described in detail previously. Pooled (15–20 buds per pool) E13.5 Eda-/- mammary buds were treated with 250 ng/ml of Fc-Edα control, and RNA was extracted, cDNA synthesized, and qRT-PCR performed with the Lightcycler480 machine (Roche, Indianapolis, IA) as described using the following primers:

- Fgf4F 5′-CGAGGAGCACTTTCTGGAG-3′, Fgf4R 5′-GTACCGGTAGGTCTTGAGG-3′, Fgf9F 5′-GGGGAGCCTTGATGAGTCA-3′, Fgf9R 5′-CCGAGGGTCTGACTG-3′, Fgf17F 5′-GACAGATACATTCGGCAGCA-3′, Fgf17R 5′-GTACGCGTAGGCTTCGTAGG-3′, Fgf20F 5′-GTGCAAGTCCAAAGAGCAT-3′, Fgf20R 5′-GGGAACTGTCTCTTGTTCT-3′. Dilution series of PCR products was used for quantifying the transcript numbers of genes of interest with the help of Lightcycler480 software. Rnbp1 (F 5′-ACCGTGGAGAGAGATGAAGA-3′, and R 5′-TCATAAGAGGGGATGTGC-3′) or GAPDH (F 5′-CTGTCGAGGCTACAAATGTCAG-3′ and R 5′-AGATGGTGAGGGGCTTCCC-3′) were used as a reference genes.

X-Gal and Carmine alum staining. X-Gal staining for whole embryos (E10.5–E15.5) or abdominal skins was performed according to a published protocol with an overnight incubation in the 1 mg/ml X-Gal substrate. For postnatal mammary glands, a modified X-Gal-staining method was used. Immunohistochemical staining was performed with in situ hybridization with digoxigenin-labeled RNA probes, E11.5–E13.5 embryos were fixed in 4% PFA overnight at 4 °C and dehydrated with rising methanol series. In situ hybridization was performed with in SituPro robot (Intavis AG) as previously published using a similar protocol. The digoxigenin-labeled RNA probes for Wnt10b, Edar, Dkk4, Lef1, PThrP, and Fgf20, have been described previously. Fgf20 probe corresponded to the open reading frame. BM Purple AP substrate Precipitating (Invitrogen) was used for detection of digoxigenin-labeled RNA probes. Radioactive in situ hybridization was performed on paraffin sections using 35S-UTP labeled (Amersham) probe specific to Fgf20 as described.

Immunohistochemical stainings. For immunohistochemical and hematoxylin-eosin stainings, the 4th mammary glands of WT and Fgf20-/-/LacZ mice were dissected, spread on microslide slides, and fixed with 4% PFA overnight at 4 °C. Alternatively, 13.5 trunks were dissected. The samples were dehydrated, embedded in paraffin, and 5 µm sections were cut. Slides were deparaffinized by standard methods. In immunohistochemical
stainings antigen retrieval was performed by heating the slides in microwave oven in TE buffer, pH 9.0 (keratin-8 (K8), keratin-14 (K14), progesterone receptor (PR) and estrogen receptor α (ERα) stainings), or in 10 mM sodium citrate buffer pH 6.0 (β-Galactosidase, cleaved Caspase-3, Ki-67, α-smooth muscle actin (α-SMA), Lef1, and p63 stainings). Primary and secondary antibodies used are listed in Supplementary information. Samples were imaged with a Zeiss Axio Imager M2 microscope equipped with an AxioCam HRC camera (Zeiss) and processed in Photoshop.

**Mammary bud area and volume quantification.** Wnt10b expression area was quantified manually from images with the help of Fiji ImageJ software. For whole-mount immunofluorescence staining E13.5 and E15.5 mouse embryos were fixed in 4% PFA at 4°C overnight. After washing the samples with PBS for 3–4 hours, they were permeabilized with 0.3% Triton-X-100 in PBS for 1–2 hours at room temperature, blocked (5% normal donkey serum, 0.5% BSA, and 0.3% Triton-X-100 in PBS) for 1 h, and incubated at 4°C with rat anti-mouse CD326 (EpCAM, BD Pharmingen, 552370, 1:1,000) and 10 µg/ml Hoechst 33342 (Molecular Probes/Invitrogen) in blocking buffer for 2 days. EpCAM was detected with an Alexa Fluor 647–conjugated secondary antibody (Molecular Probes/Invitrogen). The ventral skin around mammary gland 2 and 3 was dissected and mounted with Vectashield (Vector Laboratories) and visualized using a Zeiss LSM700 laser scanning confocal microscope. For mammary placode and bud volume quantification, the area of mammary primordium was outlined manually based on EpCAM expression and bud morphology. Surface rendering and volume quantification were performed with Imaris 8.3 software (Bitplane).

**Mammary cell preparation, cell labelling, and flow cytometry.** Single cell suspension of mammary gland was prepared according to the protocol modified from Shackleton et al. Briefly, the 4th mammary glands were cut into small pieces after removal of the lymph node. The tissues were digested in a mixture of 5 ml collagenase I buffer (10% PBS, 100 mg/ml streptomycin, 10 U/ml penicillin, 300 U/ml collagenase I (ThermoFisher) and 100 U/ml hyaluronidase (Sigma) in DMEM/F12 (1:1) medium for 1–2 hours at 37°C with moderate shaking. The cell suspension was washed in PBS and digested further in 0.25% trypsin-EDTA for 5–10 minutes. The red blood cells were removed by incubation in red blood cell lysing buffer (Biolegend) on ice for 5 minutes. The single cell suspension was passed through 40 µm cell strainer (BD Bioscience) before stained with the mixture of antibodies on ice for 30 minutes. After washing in PBS, the dead cells were labeled with Fixable Viability Dye eFluor 780 (eBioscience) for 30 minutes on ice. Flow cytometry was carried out by BD LSR II, and data analysis was performed with FlowJo. The following antibodies were used: CD45-PE (Biolegend, 103106, 1:200), CD31-PE (Biolegend, 101822, 1:200) CD24-PeCy7 (Biolegend, 101822, 1:200) and CD29-APC (eBioscience, 116207, 1:200), TER119-PE (Biolegend, 102507, 1:200), CD24-PeCy7 (Biolegend, 101822, 1:200) and CD29-APC (eBioscience, 116207, 1:200) and CD29-APC (eBioscience, 116207, 1:200).

**Monitoring the onset of puberty, estrous phase, and measurement of estradiol.** Onset of puberty was assessed by monitoring the vaginal opening (VO) by visual examination of vulva every morning 5 days/week (Mon–Fri) starting at the age of 18 days until the appearance of VO. In case of VO occurring during the weekend, the earliest, latest, and average times of VO were defined and separate comparisons of VO WTlatest vs. VO WTearliest were performed.

**Mammary fat pad transplantations.** For mammary fat pad transplantations, 3–4 week old WT (n = 6) and Fgf20LacZ/LacZ (n = 5) recipient females were anesthetized and the fat pad of left 4th mammary gland was cleared until the lymph node as described. ~1 mm3 pieces of adult (12–13-week-old) WT donor (n = 4) mammary glands were transplanted into cleared fat pads. Five weeks later transplanted mammary glands were collected, stained by Carmine alum, and ductal ends quantified.

**Statistical analysis.** P-values were calculated with unpaired t-test assuming unequal variances unless otherwise stated.

**Data availability.** The datasets generated during the current study are available from the corresponding author on reasonable request.

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Acknowledgements
We thank Raija Savolainen, Riikka Santalahti, Merja Mäkinen, Ibrahim Sultan, and Agnes Viherä for excellent technical assistance, and past and present members of the Mikkola laboratory for their help and comments. Imaging was conducted at the Light Microscopy Unit of the Institute of Biotechnology, University of Helsinki, and flow cytometry at the Flow Cytometry Core Facility in the Department of Biosciences, University of Helsinki. This work was financially supported by the Academy of Finland (grants 268798 and 272280), Finnish Cancer Foundation, Jane and Aatos Erkko Foundation, Sigrid Jusélius Foundation to MLM, NIH grant HL111190 to DMO, and NIH K99/R00 DC012825 to SHH. Fgf20−/− mice were generated with assistance from the Mouse Genetics Core, the DDRCC Murine Models Core Grant (NIH P30 DK052574), and the Washington University Musculoskeletal Research Center (NIH P30 AR057235).

Author Contributions
M.L.M. conceived the study; T.E., P.H.L., and M.L.M. designed the study; T.E., P.H.L., M.V., Q.L., E.T., C.O. performed the experiments and analyzed the data; S.H.H., D.M.O., M.P., B.A.H. provided reagents and technical assistance, and past and present members of the Mikkola laboratory for their help and comments.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-04637-1

Competing Interests: The authors declare that they have no competing interests.

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