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Estrogen modulates mesenchyme-epidermis interactions in the adult nipple

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Abstract

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Maintenance of specialized epidermis requires signals from the underlying mesenchyme; however, the specific pathways involved remain to be identified. By recombining cells from the ventral skin of the *K14-PTHrP* transgenic mice [which overexpress parathyroid hormone-related protein (PTHrP) in their developing epidermis and mammary glands] with those from wild type, we show that transgenic stroma is sufficient to reprogram wild-type keratinocytes into nipple-like epidermis. To identify candidate nipple-specific signaling factors, we compared gene expression signatures of sorted *Pdgfra*-positive ventral *K14-PTHrP* and wild-type fibroblasts, identifying differentially expressed transcripts that are involved in WNT, HGF, TGF β , IGF, BMP, FGF and estrogen signaling. Considering that some of the growth factor pathways are targets for estrogen regulation, we examined the upstream role of this hormone in maintaining the nipple. Ablation of estrogen signaling through ovariectomy produced nipples with abnormally thin epidermis, and we identified TGF β as a negatively regulated target of estrogen signaling. Estrogen treatment represses Tgf β 1 at the transcript and protein levels in *K14-PTHrP* fibroblasts *in vitro*, while ovariectomy increases *Tgfb1* levels in *K14-PTHrP* ventral skin. Moreover, ectopic delivery of Tgf β 1 protein into nipple connective tissue reduced epidermal proliferation. Taken together, these results show that specialized nipple epidermis is maintained by estrogen-induced repression of TGF β signaling in the local fibroblasts.

KEY WORDS: Fibroblast, Nipple, Estrogen receptor α , TGF β , Epidermis

INTRODUCTION

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Vertebrates interact with and manipulate their environment by using regions of specialized skin. Epidermis in such specialized skin sites often has distinct stratification patterns, expresses unique differentiation markers and features novel appendages ([Billingham and Silvers, 1967](#)). In humans, such sites include

nipples, lips, palms, soles, anal and genital skin ([Schweizer et al., 1984](#)). Mice share these sites and also feature distinct tail, muzzle and ear skin ([Schweizer, 1993](#)). Among these, the nipple stands out for its pivotal role in the mammalian life cycle as a key site for milk transfer from mother to offspring. Thickened and highly proliferative nipple epidermis is uniquely adapted to withstand high mechanical and frictional forces associated with milk delivery ([Eastwood et al., 2007](#); [Koyama et al., 2013](#); [Mahler et al., 2004](#)), and it expresses a unique set of keratins and intermediate filament-associated proteins that are largely absent in the trunk epidermis ([Eastwood et al., 2007](#); [Mahler et al., 2004](#)). Despite its important function and unique characteristics, little is known about how this epidermal differentiation state is acquired and maintained.

Generally, epithelial specialization is thought to require inductive signals from the underlying connective tissue ([Dhouailly et al., 1998](#)). For example, seminal experiments conducted by the Billingham group found that grafts of sole dermis recombined with ear or trunk epidermis produced a thickened and expanded cornified layer, indicating that the mesenchyme specifies the phenotype of the grafted epithelium ([Billingham and Silvers, 1967](#)). In other classic experiments, recombination of the palatal epithelium with the cheek connective tissue or vice versa resulted in the *de novo* keratin expression pattern normally associated with the connective tissue type ([Mackenzie and Hill, 1981, 1984](#); [Schweizer et al., 1984](#)). Additionally, grafting of the non-palmoplantar epidermal cells onto injured human soles led to epidermis adopting a palmoplantar phenotype, complete with the expression of the site-specific keratin K9 ([Yamaguchi, 1999](#)). The lead role of fibroblasts in determining the palmoplantar characteristics of the associated epidermis was further confirmed in follow-up experiments ([Yamaguchi, 1999](#)). These classic studies suggest a central role for fibroblasts in determining the anatomical specificity of epidermal cell fate and characteristics.

The inductive and maintenance growth factors produced by site-specific and appendage-specific skin fibroblasts are just starting to be defined ([Driskell et al., 2013](#); [Driskell and Watt, 2015](#); [Sriram et al., 2015](#)). Perhaps, the most well-studied cells in this respect are the dermal papilla fibroblasts of the hair follicle, which induce both the formation ([Jahoda et al., 1984, 1993](#); [Plikus, 2014](#); [Yang and Cotsarelis, 2010](#)) and regenerative cycling of hair follicles ([Chi et al., 2013](#); [Clavel et al., 2012](#); [Enshell-Seijffers et al., 2010](#); [Morgan, 2014](#); [Rendl et al., 2005](#); [Sennett and Rendl, 2012](#)). Dermal papilla fibroblast-specific factors include the fibroblast growth factors Fgf7 and Fgf10 ([Chi et al., 2013](#); [Clavel et al., 2012](#); [Enshell-Seijffers et al., 2010](#); [Morgan, 2014](#); [Rendl et al., 2005](#); [Sennett and Rendl, 2012](#)), bone morphogenetic proteins Bmp4 and Bmp6 ([Clavel et al., 2012](#); [Rendl et al., 2005, 2008](#)), the BMP antagonist noggin ([Botchkarev et al., 1999, 2002](#); [Rendl et al., 2005](#)), transforming growth factor TGF β 2 ([Oshimori and Fuchs, 2012](#)) and many others ([Morgan, 2014](#); [Rendl et al., 2005](#); [Sennett and Rendl, 2012](#)). Outside of the hair follicle, skin fibroblasts also feature significant specialization and heterogeneity; however, our understanding of their molecular profiles is still rudimentary ([Driskell et al., 2013](#); [Driskell and Watt, 2015](#); [Sriram et al., 2015](#)). For instance, recent studies show that papillary (upper) dermis fibroblasts express higher levels of growth factors that control epidermal proliferation and differentiation, whereas those from the reticular (lower) dermis produce high levels of signaling molecules associated with matrix production ([Driskell et al., 2013](#); [Driskell and Watt, 2015](#); [Sriram et al., 2015](#)). These findings imply that transcriptional heterogeneity is an intrinsic property related to the developmental history of specific fibroblasts. The notion is further supported by transcriptional profiling studies on skin fibroblasts from different anatomical locations ([Chang et al., 2002](#); [Rinn et al., 2006, 2008](#)). Signature profiles of regionally specific skin fibroblasts are in part maintained by their unique homeobox (HOX) gene activities. For example, in human skin, the HOXB genes are expressed in the trunk and non-dermal fibroblasts, whereas HOXD4 and HOXD8 are found exclusively in trunk and proximal leg fibroblasts. Intriguingly, HOXA13 is limited in expression to fibroblasts in distal body sites including hands, feet and foreskin, and its activity is required for the expression of the distal-specific Wingless-Int family growth factor WNT5A ([Chang et al., 2002](#); [Rinn et al., 2006, 2008](#)). In mice, differential expression of the HOX gene *Tbx15* between dorsal and

ventral skin fibroblasts contributes to the differential skin pigmentation along the sagittal body axis (Candille et al., 2004). Taken together, these findings imply that the developmental history of fibroblasts profoundly impacts upon their inductive and maintenance interactions with the epidermis.

The specific developmental origin of the nipple connective fibroblasts that lie underneath the structure has not been completely established. The mammary gland develops at the interface of what will become dorsal and ventral skin. The mesenchymal cells that lie underneath the mammary line at embryonic day (E)10.5 may be derived from the hypaxial mesoderm (Dhouailly and Oftedal, 2016; Oftedal and Dhouailly, 2013). However, it is not clear whether these or other cells condense around the bud as the primary mammary mesenchyme at E11 to E12.5 (Sakakura, 1987; Sakakura et al., 1987). The differentiation of the mammary mesenchyme is driven in part by parathyroid hormone-related protein (PTHrP; also known as PTHLH) signaling from the developing epithelial cells via PTH/PTHrP receptor (also known as PTH1R) expressed on the stroma. In turn, inductive signaling from these differentiated fibroblasts is required to produce the nipple sheath, which is of distinct evolutionary origin from the gland (Oftedal and Dhouailly, 2013) at E17 (Dunbar et al., 1999; Dunbar and Wysolmerski, 1999; Wysolmerski et al., 1998). Intriguingly, ectopic expression of PTHrP driven by the human keratin 14 promoter in *K14-PTHrP* mice (hereafter KrP mice) dramatically expands the differentiation of the lateral plate mesoderm derivatives to the mammary mesenchyme fate. However, this has minimal impact on cells of the dorsal dermis (Foley et al., 2001). In part, this appears to be the result of increased *Bmp4* signaling in the developing ventral skin of the embryo, driving mammary mesenchyme differentiation (Hens et al., 2007). However, other factors such as timing of transgene expression or sensitivity of somatic versus lateral plate-derived mesenchymal cells to PTHrP may be involved (Foley et al., 2001). In the adult female mouse, the ectopic expression of PTHrP results in a hairless skin with a thickened epidermis and complex connective tissue consistent with the nipple (Abdalkhani et al., 2002; Foley et al., 2001, 1998). Whether the fibroblasts that underlie the nipple are simply derived from mammary mesenchyme cells remains to be determined. Nevertheless, during skin development these cells appear to have the capacity to induce and maintain the specialized epidermis that characterizes the structure (Foley et al., 2001; Wu et al., 2015).

In this study, we set out to define the inductive and signaling properties of the specialized fibroblasts from the nipple skin. We established that KrP fibroblasts robustly induce reprogramming of the trunk epidermis towards nipple fate. We also established the unique transcriptional signature of the KrP fibroblasts and evaluated the impact of key pathways identified by that analysis.

RESULTS

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Fibroblast-induced reprogramming of trunk keratinocytes into nipple-like epidermis

The instructive capacity of highly specialized nipple fibroblasts has not been established as studies have been limited by the very small size of the appendage in mice. To determine whether nipple fibroblasts have the capacity to reprogram the fate of trunk epidermis, a grafting experiment was carried out using purified cells from ventral skin of neonatal female KrP mice (Wysolmerski et al., 1994), in which close to one quarter of the entire body skin is nipple like (Foley et al., 2001). KrP fibroblasts were recombined with wild-type (WT) neonatal trunk keratinocytes from both dorsal and ventral skin. The resulting grafts were compared to those reconstituted from WT epidermal and dermal cells, as well as the ventral skin of KrP mice (Fig. 1A) (Lichti et al., 2008). When WT keratinocytes and WT fibroblasts were grafted, hairy skin was produced, as previously reported ($n=3$) (Fig. 1B, left) (Lee et al., 2011; Lichti et al., 2008, 1995, 1993). As expected, reconstitution of KrP neonatal keratinocytes with KrP fibroblasts produced pigmented nipple-like skin with a thickened epidermis and without hair follicles ($n=3$) (Fig. 1B,C, right panels). Importantly, grafts that recombined WT neonatal keratinocytes with KrP fibroblasts also resulted in pigmented nipple-like skin ($n=3$) with similar histology to those reconstituted from KrP cells only (Fig. 1B, third panel and 1

C, middle panel). As shown on [Fig. 1D,E](#), grafts that contained KrP ventral fibroblasts expressed the nipple epidermis-specific marker keratin K2e (also known as KRT2) and had expanded filaggrin expression, whereas grafts with only WT cells did not. In normal haired mouse skin, melanocytes localize exclusively to hair follicles ([Quevedo and Fleischmann, 1980](#)). In contrast, connective tissue of grafts based upon ventral KrP dermal cells contained pigmented cells that we have previously determined to be melanocytes ([Abdalkhani et al., 2002](#)) ([Fig. 1C](#), arrows). Thus, dermal cells from KrP ventral skin produce a signaling environment sufficient to induce reprogramming of neonatal trunk keratinocytes towards nipple-like epidermis and formation of pigmented connective tissue.

Pdgfra is a marker for nipple fibroblasts

Platelet-derived growth factor receptor α (Pdgfra) was previously reported to be a useful marker for isolating dermal fibroblasts from haired skin ([Collins et al., 2011](#)). Here, by using immunofluorescence, we show that Pdgfra is expressed in nipple fibroblasts, but not in basal keratinocytes in WT ventral skin. It is also expressed in ventral dermis of KrP mice ([Fig. 2A](#)). Next, we evaluated Pdgfra staining along with staining for the fibroblast marker vimentin and found substantial overlap ([Fig. 2B](#)). Dual staining for the smooth muscle marker Acta2 and Pdgfra revealed co-expression in blood vessels, arrector pili muscles, smooth muscle-like cells along the lactiferous duct of the nipple and nests of contractile cells within KrP ventral dermis. These data indicate that Pdgfra can be used as a marker for isolating specialized fibroblasts from KrP ventral skin; however, the Pdgfra-positive fraction will also include a few Acta2-positive vascular and muscle cells.

Next, fibroblasts were isolated by fluorescence-activated cell sorting (FACS) from the nipple-like skin of virgin adult female KrP mice and ventral skin of their WT littermates by using an anti-Pdgfra antibody (which labels the CD140 epitope) ([Fig. 3A](#)). To purify cells expressing high amounts of CD140 (the CD140^{hi} population), we set up a dump channel to exclude cells expressing CD31 (endothelial cells), CD117 (melanocytes), CD45 (hematopoietic cells) or CD49f markers (keratinocytes) ([Collins et al., 2011](#)). Next, we harvested both WT and KrP RNAs from three distinct cell populations: (1) CD140^{hi} and dump⁻ cells, (2) CD140⁻ and dump⁺ cells, and (3) viable cells expressing none of the markers ([Fig. 3B](#)). There was also almost a twofold increase of *Pdgfra* and an elevated expression of *Vim* and *Colla2* in KrP compared to WT CD140⁺ sorted cells. Next, sorted CD140⁺ or dump⁺ cell populations were transiently cultured for 48 h. While cultured CD140⁺ cells had a branched and elongated spindle-like shape, morphologically similar to a typical fibroblast, dump⁺ cells, were mostly epithelial in appearance (compare [Fig. 3C](#) versus [Fig. 3D](#)) ([Kalluri and Zeisberg, 2006](#)). Thus, we confirmed that Pdgfra is highly expressed in dermal fibroblasts from the ventral skin of both WT and KrP female mice and that it can be used as a marker for their isolation by sorting.

Microarray profiling reveals a unique gene expression signature of KrP fibroblasts

To identify candidate inductive and maintenance factors present in nipple fibroblasts, we performed microarray-based transcriptome profiling on FACS-sorted CD140⁺ fibroblasts from KrP and WT ventral skin. A total of 123 genes were upregulated and 118 were downregulated in KrP as compared to ventral WT fibroblasts. To identify signaling pathways altered in KrP fibroblasts, differentially expressed genes were subjected to Gene Ontology (GO) analysis and altered signaling of WNT, HGF, TGF β , IGF, BMP and FGF pathway elements were identified ([Fig. 4](#); [Fig. S1](#)). Interestingly, selected extracellular matrix transcripts, including *Colla1* and *Colla2*, as well as those from the Timp family of metalloproteinase inhibitors were upregulated, while several metalloproteinase-encoding genes were downregulated in the KrP fibroblasts.

The differential expression of 30 transcripts (20 increased, six decreased and four unchanged in KrP

relative to WT fibroblasts), many of which are part of the pathways identified by the Ingenuity and GO term analysis, was largely confirmed by quantitative real-time RT-PCR (qRT-PCR) on sorted fibroblasts as well as micro-dissected virgin WT nipples, WT and intact KrP ventral skin (Figs S2 and S3). We grew both WT and KrP ventral fibroblasts *in vitro* for up to five passages and found signature genes remained differentially regulated in KrP relative to WT ventral fibroblasts (Fig. S4). Overall, differential expression of most genes that comprise the KrP fibroblast signature are known to be consistent in the nipple and stable in primary culture (Rinn et al., 2006, 2008).

Estrogen receptor signaling in fibroblasts maintains nipple structure

Fibroblasts associated with the mammary gland in mice have long been recognized to be responsive to estrogen (Hiremath et al., 2012; Cunha et al., 1997; Wu et al., 2015). Not surprisingly, pregnancy- and lactation-related hormone receptors, including progesterone receptor (*Pgr*), oxytocin receptor (*Oxtr*) and relaxin receptor (*Rxfp1*) were strongly upregulated in the nipple-like KrP fibroblasts as determined by microarray analysis (Fig. 5A). Since both oxytocin and progesterone receptors are regulated by estrogen (Lapidus et al., 1998; Zingg et al., 1998), we next performed qRT-PCR analysis on these hormone receptor-regulated genes using intact ventral skin as well as sorted fibroblasts. Indeed, transcripts for *Esr1*, *Oxtr* and *Pgr* were significantly increased in the WT nipple and KrP nipple-like ventral skin relative to WT non-nipple skin (Fig. 5B). Similar results were also observed for the CD140⁺ sorted KrP fibroblasts (Fig. 5B). Focusing on *Esr1*, its differential expression between primary cultured fibroblasts from WT versus KrP virgin female ventral skin was confirmed at the protein level by western blotting (Fig. 5C) and in the intact WT nipple by immunofluorescence (Fig. 5D). At the cellular level, nipple fibroblasts had substantial nuclear *Esr1* expression (Fig. 5D) (McCormack and Greenwald, 1974). These findings suggest that nipple fibroblasts, including these from the KrP mouse model, maintain stable *Esr1* expression and are the target sites for estrogen action.

To further investigate the role of ovarian hormones in the nipple, we ovariectomized (ovexed) pre-pubertal WT and KrP mice, and then harvested nipples and KrP ventral skin between 1.5 and 3 months later. We found that the nipple epidermis in ovexed mice is much thinner than in controls, and the basal layer is less markedly invaginated (Fig. 5E). These changes were accompanied by the diminished expression of the nipple epidermal markers K2e and filaggrin (Fig. 5F; Fig. S5). Moreover, nipple size, which is largely controlled by the extracellular matrix (Wu et al., 2015), was ~30% smaller in the ovexed WT mice as compared to age-matched controls, and specialized nipple connective tissue, typically composed of small tightly packed collagen bundles, was markedly diminished in the ovexed KrP mice (Fig. 5E). Thus, the removal of ovarian hormones, including estrogens, impacts upon both the epidermis and the connective tissue of the nipple.

TGFβ signaling is downregulated in KrP fibroblasts

Upon reviewing the growth factor pathways differentially represented in the KrP fibroblasts as determined by the GO analysis, we found that several of them can be regulated by estrogen in non-nipple tissues (Hewitt et al., 2010; Knabbe et al., 1987; Yokota et al., 2008). Among these is the TGFβ pathway, which has been shown to be inhibited by estrogen signaling components at multiple levels (Cherlet and Murphy, 2007; Colletta et al., 1990), including repression of ligand secretion (Knabbe et al., 1987). This prompted us to further evaluate Tgfβ1 production and signaling in the context of nipple tissue. Indeed, the level of *Tgfb1* transcript was decreased by 50% in sorted KrP fibroblasts as compared to WT ventral skin fibroblasts (Fig. 6A). A similar decrease, of ~70%, was found in intact WT nipple and KrP skin as compared to ventral WT skin of virgin mice (Fig. 6A). To further validate the reduced TGFβ signaling, we examined the expression levels of phosphorylated Smad2 and Smad3 (pSmad2/3), a specific downstream marker of TGFβ signaling. We show that while nuclear pSmad2/3 expression was observed in most of the

ventral skin fibroblasts, fewer nipple fibroblasts were positive (Fig. 6B). Secreted Tgfb1 protein (as measured by ELISA) was reduced by ~50% at 48 or 72 h of culture of primary KrP versus WT ventral skin fibroblasts (Fig. 6C), while *Tgfb1* transcript expression remained stably reduced in cultured primary KrP fibroblasts (Fig. 6C) for at least four passages (not shown). As seen by western blotting, the amount of pSmad2/3, but not that of total Smad2/3 protein was reduced in KrP versus WT cultured fibroblasts (Fig. 6D). Taken together, these data suggest that TGFβ signaling is downregulated in nipple fibroblasts.

Estrogen regulates *Tgfb1* transcript levels in KrP fibroblasts

To further establish a functional relationship between estrogen and TGFβ signaling in nipple fibroblasts, we employed primary fibroblast culture in stripped serum Phenol Red-free medium. WT or KrP fibroblasts were treated with 0.5 or 10 nM estradiol for 48 or 72 h, and were then subjected to qRT-PCR analysis. For KrP fibroblasts culture, removal of Phenol Red, a known weak estrogen mimetic, resulted in an increase in the level of *Tgfb1* transcript by 50%, whereas addition of 0.5 nM estradiol lowered *Tgfb1* to the baseline level and 10 nM estradiol reduced it by another 50% (Fig. 6E). We also validated the effect of estradiol on Tgfb1 at the protein level by ELISA measurements. While the serum-free conditions reduced total Tgfb1 levels in both WT and KrP fibroblasts, addition of 10 nM of estradiol decreased the amount of protein by 40% only in KrP fibroblast cultures (Fig. 6F). Taken together, these *in vitro* assays demonstrate that *Tgfb1* production is specifically repressed by exogenous estradiol in the KrP fibroblast.

Next, we evaluated TGFβ signaling in an *in vivo* system with altered estrogen signaling, the ovexed KrP mice. As shown in Fig. 6G, *Tgfb1* transcript levels were elevated fourfold in the ventral skin of KrP mice that had been ovexed for 16 weeks, whereas the estrogen-regulated *Esr1* and *Pgr* were reduced by 50% as compared to age-matched samples from intact female KrP mice. As assessed by western blotting, pSmad2/3 protein was elevated in ovexed versus non-ovexed KrP mice (Fig. 6H; Fig. S6), indicating that signaling downstream of TGFβ was elevated in KrP skin under conditions of low estrogen.

Dermal but not epidermal Tgfb1 partially shifts nipple features towards those of trunk skin

To initially test the impact of Tgfb1 on nipple skin, beads that slowly release Tgfb1 were implanted into 8-week-old virgin female mouse skin. Beads with or without 0.2 μg of murine recombinant Tgfb1 were implanted into the nipple dermis of WT mice or ventral skin of KrP mice and their effect was analyzed after 7 days. In contrast to buffer-treated control beads, Tgfb1-treated beads had a slightly thinner epidermis in WT nipple and KrP skin and led to a thinner papillary dermal layer in the transgenic mice (Fig. 7C). This was accompanied by a significant decrease in epidermal BrdU labeling by ~50%. In this experiment, Tgfb1 did not have a measurable impact on WT non-nipple skin (Fig. 7C). To investigate whether Tgfb1 overexpression could directly influence nipple epidermis, we used doxycycline to induce transgene expression in the *K14-rTA/tetO-TGFb1* mice (Liu et al., 2001), which produces a non-latent porcine form of the TGFβ1 ligand. As shown in Figs S7 and S8, nipple epidermal thickness and BrdU incorporation were similar in transgene-expressing and non-induced double transgenic mice, whereas BrdU incorporation was substantially reduced in the ventral skin of TGFβ1-overexpressing mice. Taken together, these results suggest that overexpression of TGFβ1 within the dermis rather than the epidermis has a greater impact on nipple skin.

DISCUSSION

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In this work, we have defined the key molecular and cellular attributes of the fibroblast population that is underneath the specialized epidermis of the murine nipple. First, we showed that fibroblasts from newborn KrP mice induce reprogramming of WT trunk skin keratinocytes towards the nipple fate. Second, we identified the unique gene expression signature of the KrP fibroblasts. Next, we showed that *Esr1* signaling is among the major pathways active in KrP fibroblasts and that it acts to suppress TGFβ signaling in KrP

fibroblasts *in vitro*. We also present evidence suggesting that the reduced TGF β pathway activity is essential for endowing nipple skin with its unique characteristics in the adult.

To date, palmoplantar fibroblasts are the most well-defined example of site-specific skin fibroblasts. Their ability to induce reprogramming of keratinocytes into thickened epidermis with a unique keratin expression pattern ([Yamaguchi, 1999](#)) has been attributed to several secreted factors. One of them is Dkk1, a soluble inhibitor of canonical WNT signaling ([Yamaguchi et al., 2004, 2009, 2008](#)), which also functions to reduce pigmentation of palms and soles by inhibiting melanocyte activity ([Yamaguchi et al., 2004, 2009](#)). Another factor is the non-canonical WNT ligand Wnt5a, a putative downstream target of Hoxa13 ([Rinn et al., 2006, 2008](#)). Here, we provide the first insight into the signaling network that defines site-specific properties of nipple fibroblasts.

Our recombination experiments confirm the leading role of fibroblasts in endowing skin with its regional specificity. We show that KrP stromal cells reprogram WT trunk skin keratinocytes to have nipple-like features, including its stratification pattern and marker profile. It is noteworthy that our KrP stromal cells grafts were pigmented, similar to WT nipple and intact KrP ventral skin ([Abdalkhani et al., 2002](#)). This suggests that fibroblasts regulate site-specific skin pigmentation pattern, consistent with the previous experiments on palmoplantar fibroblasts ([Yamaguchi et al., 2004, 2009](#)).

Commonly, specialized skin sites in mammals undergo physiological changes in adult life. For instance, palmoplantar skin responds to weight bearing by altering epidermal proliferation dynamics to produce a thick, protective callus ([Menz et al., 2007](#)). Perineal skin in the anogenital region of the Old World monkeys can dramatically change in synchrony with the altered reproductive states ([VandeBerg et al., 2009](#)). The human nipple and areola skin increases in size and pigmentation during pregnancy and lactation ([Javed and Lteif, 2013; Neifert et al., 1990](#)). Estrogen is present at moderate levels during nipple morphogenesis and *Esr1* is dispensable for its formation ([Bocchinfuso and Korach, 1997](#)). Our new data from the ovariectomized mice indicate that estrogen signaling in fibroblasts is necessary for maintaining the thickened epidermis and some connective tissue features of the adult virgin nipple ([Fig. 5](#)). The nipple expands during late pregnancy, when estrogen levels peak, and this is accompanied by high levels of nuclear *Esr1* in the fibroblasts ([Wu et al., 2015](#)). This prompted us to speculate that estrogen likely triggers changes in the signaling environment required for the production and expansion of adult nipple connective tissue, and the maintenance of the unique thickened epidermis. Among several candidate pathways identified in our transcriptomic studies, we focused on the reduction of the TGF β signaling because it has known effects on fibroblast biology, including regulation of extracellular matrix production, and modulation of growth factor secretion, including keratinocyte growth factor and colony stimulating factor *Csf1*, which are crucial for the regulation of epidermal proliferation and differentiation ([Mauviel, 2009; Szabowski et al., 2000](#)). Consistent with this, we now show that short-term Tgf β 1 bead implantation into the WT nipple and KrP ventral dermis reduces epidermal proliferation, whereas epidermal overexpression of the growth factor fails to do so. One caveat to these Tgf β 1 studies is that they both used a simple ligand in contrast to the more complex endogenous form, which involves the latency-associated peptide as well as latent TGF β -binding proteins ([Robertson et al., 2015](#)). Nevertheless, these findings imply that at least some aspects of the adult nipple epidermal phenotype can be reversed by ectopic activation of TGF β signaling, and this appears to be an indirect effect of reduced autocrine TGF β activity in nipple fibroblasts ([Mauviel, 2009](#)). We posit that further studies on some of the signaling pathways revealed here could lead to novel regenerative and tissue engineering approaches for nipple replacement.

MATERIALS AND METHODS

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Mice

The *K14-PThrP* (KrP) transgenic line ([Wysolmerski et al., 1994](#)) was maintained by continual breeding

against unrelated C57BL/6 mice. WT mice used in this study were either inbred C57BL/6 or littermates of the KrP mice. All animal use was approved by the Indiana University Institutional Animal Care and Use Committee, and was performed in compliance with stipulations of that body. *K14-rTA/tetO-TGF β 1* mice were fed 1 g/kg body weight doxycycline chow (Bio-Serve) for 3 weeks to induce transgene expression. Nipples and skin were harvested after BrdU injection (see below). Inbred 1.5- to 3-month-old *Foxn1^{nu/nu}* BALB/c mice (NU/J Jackson Labs) were used as recipients of grafts.

Grafting

Pups derived from the mating of KrP male and WT female mice (1 to 2 days old) were used to isolate keratinocytes and fibroblasts following the protocols developed by Lichti, Yuspa and colleagues ([Lichti et al., 2008](#), [1995](#), [1993](#); [Weinberg et al., 1993](#)). After genotyping, the ventral skin used was exclusively from KrP-positive female pups whereas the entire trunk skin was used from WT pups. The grafts were composed of 10^7 fibroblasts and 1×10^6 - 2×10^6 keratinocytes. These were mixed together and applied onto Integra[®] Dermal Regeneration Template (Integra) and incubated for 1 to 2 h prior to placing on the mouse. The grafting procedure followed the method of [Lee et al. \(2011\)](#). Grafts were harvested 2 to 4 months after the surgery. For each round of grafts, WT epidermal and dermal cells were grafted alone to ensure that hair shafts were not produced, which indicated purity of the cellular preparations (not shown).

Isolation of adult fibroblasts

Primary fibroblasts were isolated as previously described ([Lichti et al., 2008](#); [Wu et al., 2015](#)). Briefly, ventral skin from 6- to 8-week-old female virgin WT or KrP mice was carefully dissected to remove mammary glands, and floated on 0.25% trypsin (Gibco Thermo Fisher Scientific) overnight at 4°C. The next day, epidermis was removed, and the remaining tissue was minced and enzymatically digested in a collagenase mixture. The retained cells were then centrifuged at 150 *g* for 5 min, and the supernatant was further pelleted at 450 *g* for 5 min. Next, pellets from both the 150 *g* and 450 *g* spin-down were pooled together and washed with Dulbecco's modified Eagle's medium (DMEM) twice, then centrifuged at 230 *g* for 5 min before being strained through a 45 μ m mesh. The retained cells were suspended in DMEM and plated at 10^6 cells per 100 mm dish. Additional information on media and chemicals used for this process can be found in the [supplementary Materials and Methods](#).

Flow cytometry

The isolated cells were labeled with anti-mouse CD140a-APC (17-1401-81, eBioscience), anti-mouse CD31-(PECAM-1)-PE (12-0311, eBioscience), anti-mouse CD45-PE (12-045, eBioscience), anti-mouse CD117-(c-kit)-PE (12-1171, eBioscience) and anti-human/anti-mouse CD49f-(Integrin α 6)-PE (12-0495, eBioscience) ([Collins et al., 2011](#)) (antibodies were used according to the manufacturer's instruction). Meanwhile, 7-aminoactinomycin D (7-AAD) viability staining was performed to exclude dead cells. Cells without antibody labeling and cells with only secondary antibody labeling were used as controls to set the gates. CD140⁺ cells were collected by using the flow cytometry sorter with FACSDiva software, FACSAria II (BD Biosciences) sorter. FACS analysis was evaluated by FlowJo version X (FlowJo LLC). Re-analysis of the sorted cells indicated a 96-98% purity of CD140⁺ cells.

Gene expression profiling and microarray studies

Total RNA from CD140⁺/dump⁻ dermal fibroblasts from WT or KrP ventral skin of three independent groups, each using five WT and three *K14-PTHrP* female mice were purified as described above. RNA quality was measured by using a 2100 Bioanalyzer (Agilent Technologies), and RNA quantity was accessed by using a Qubit[®] 2.0 Fluorometer ([Q32866](#), Thermo Fisher Scientific). 20 ng of RNA with RNA integrity number (RIN) \geq 8 were subjected to reverse transcription and hybridization to Affymetrix Mouse

Gene 2.0 ST Arrays. Gene expression data was processed and analyzed using Affymetrix Microarray Analysis Suite Version 5.0 (MAS5.0). Data are available at the Gene Expression Omnibus database under accession [GSE87030](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87030) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87030>). To identify differentially expressed genes, gene expression was compared between the WT and KrP group in each experiment using a cut-off value of $P < 0.05$ in a two-tailed t -test and with a fold change of over twofold. Principal Component Analysis was conducted and the Volcano plot produced with the R software package. Differentially expressed genes were then subjected to hierarchical clustering as well as pathway prediction analysis using Network2Canvas ([Tan et al., 2013](#)). See [Table S1](#) for individual transcript data.

ELISA for Tgfb β 1

5×10^5 WT or KrP primary fibroblasts were seeded in 10 cm dishes in DMEM growth medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μ g/ml streptomycin overnight for attachment and then serum starved for 12 h. Supernatants were collected at 48 h and 72 h after incubation and centrifuged at 16,000 g for 10 min at 4°C to remove any debris and stored at -80°C until used. Samples were measured using mouse Tgfb β 1 Quantikine ELISA (MB-100B, R&D Systems) following the manufacturer's instruction. Cells were counted after supernatants were harvested, and the Tgfb β 1 measurements were normalized to the cell number. Each experiment was carried out in triplicate.

Tgfb β 1 agarose bead preparation and implantation

Recombinant mouse Tgfb β 1 (7666-MB-005, R&D Systems, Minneapolis, MN) was lyophilized with 0.1% bovine serum albumin (BSA) and stored at -20°C until use. 4 mM of HCl was used to reconstitute recombinant Tgfb β 1 to a final concentration of 5-10 μ g/ml. 250 μ l of Affi-Gel Blue beads (100-200 mesh, Bio-Rad) were soaked in 250 μ l of 10 μ g/ml Tgfb β 1 and incubated at 37°C for 1 h. Beads soaked with lyophilized BSA reconstituted in 4 mM HCl were used as controls. 150 μ l of agarose bead suspensions were then injected into the ventral skin of WT and KrP 8-week-old mice using a 22-gauge needle. Sites of agarose bead implantation and the surrounding area of the implantation were harvested after 7 days.

Histology and immunofluorescence

Samples were collected, fixed and sectioned as previously described ([Wu et al., 2015](#)). Fluorescence microscope images were obtained with a Nikon NiE (Nikon Instruments) and confocal images were obtained with a Leica TCS SP5 laser-scanning confocal microscope (Leica Microsystems), both at the IUB Light Microscopy Imaging Center of Indiana University. Additional information on antibodies and reagents used for this process can be found in the [supplementary Materials and Methods](#).

Tissue protein extraction and western blotting

Hair-free ventral skin was removed from KrP and KrP ovexed mice. Skin samples were snap-frozen using liquid nitrogen. Samples were digested in a 4% SDS, protease and phosphatase inhibitor solution (1:100, #P8340 and #P0044, Sigma-Aldrich). Mechanical lysis was carried out using an Eppendorf pestle followed by a QIAshredder column. The supernatant protein concentration was measured by using the Pierce BCA protein assay kit (#23227, Thermo Fisher Scientific). Samples were diluted to 0.35 μ g/ml, 0.70 μ g/ml or 1.40 μ g/ml using the 4% SDS solution and a Laemmli Buffer. Whole-cell lysates from primary fibroblasts were prepared by using RIPA buffer, and were simultaneously monitored by blotting for ESR1, pSMAD2/3, SMAD2/3 and tubulin, as a loading control, to determine that increased estrogen receptor levels were correlated with reduced Tgfb β 1 signaling *in vitro*. Western blotting was carried out as previously described ([Nickerson, 2013](#)). Additional information on antibodies and reagents used for western blotting can be found in the [supplementary Materials and Methods](#).

qRT-PCR

Total RNA was isolated from sorted cells and/or tissues as previously described (Wu et al., 2015) by using an RNeasy mini kit (74104 and 74106, QIAGEN) or an RNeasy micro kit (74004, QIAGEN) with on-column DNase I treatment according to manufacturer's instruction. 1 µg of total RNA was reverse transcribed using SuperScript III First-Strand Synthesis SuperMix Kit (18080400, Thermo Fisher Scientific) to generate cDNA. 40 ng of cDNA was used for qRT-PCR performed on a CFX Connect real-time system (Bio-Rad) using iTaq Universal SYBR Green Supermix (172-5124, Bio-Rad). All results were normalized to the *Gapdh* housekeeping gene using $\Delta\Delta C_T$ method. Primer sequences are available from (Bio-Rad). Analysis was performed on triplicates from samples of cDNA from five animals. Analyses were repeated twice with independently sourced RNA.

BrdU incorporation

Proliferation was assessed by BrdU incorporation through intraperitoneal injection 4 h prior to tissue harvest. A stock solution of 20 mg/ml of 5-bromo-2'-deoxyuridine (BrdU; B5002, Sigma-Aldrich) in 0.9% saline was prepared and stored at -20°C until use. 1 mg of BrdU was injected per 100 g body weight of the mice (Wu et al., 2015).

Statistics

Unless indicated, all *in vivo* data are presented as mean±s.e.m. from at least three replicates from three to six individual animals. All *in vitro* data are presented as mean±s.d. of triplicate measurements of three independent replicates for a single experiment. Experiments were repeated at least twice. Comparisons were evaluated with a Student's *t*-test by using GraphPad Prism, version 5.0 (GraphPad Software); *P*<0.05 was considered to be significant. The genome-wide analysis was carried out using Partek Genomic Suite, version 6.5 (Partek).

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Footnotes

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.-J.W., A.B.G., D.F.S., C.D.O., S.T., S.K. and J.F. designed the experiments. H.-J.W., D.F.S., S.T., S.K., C.D.O., L.J.S. and J.F. performed the experiments. H.-J.W., J.F., J.D., M.V.P., S.K., and J.W.O. analyzed the data and H.-J.W., M.V.P., S.K. and J.F. wrote the manuscript.

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Data availability

The microarray data reported in this study has been deposited in the Gene Expression Omnibus database under accession number [GSE87030](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87030) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87030>).

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.141630.supplemental>

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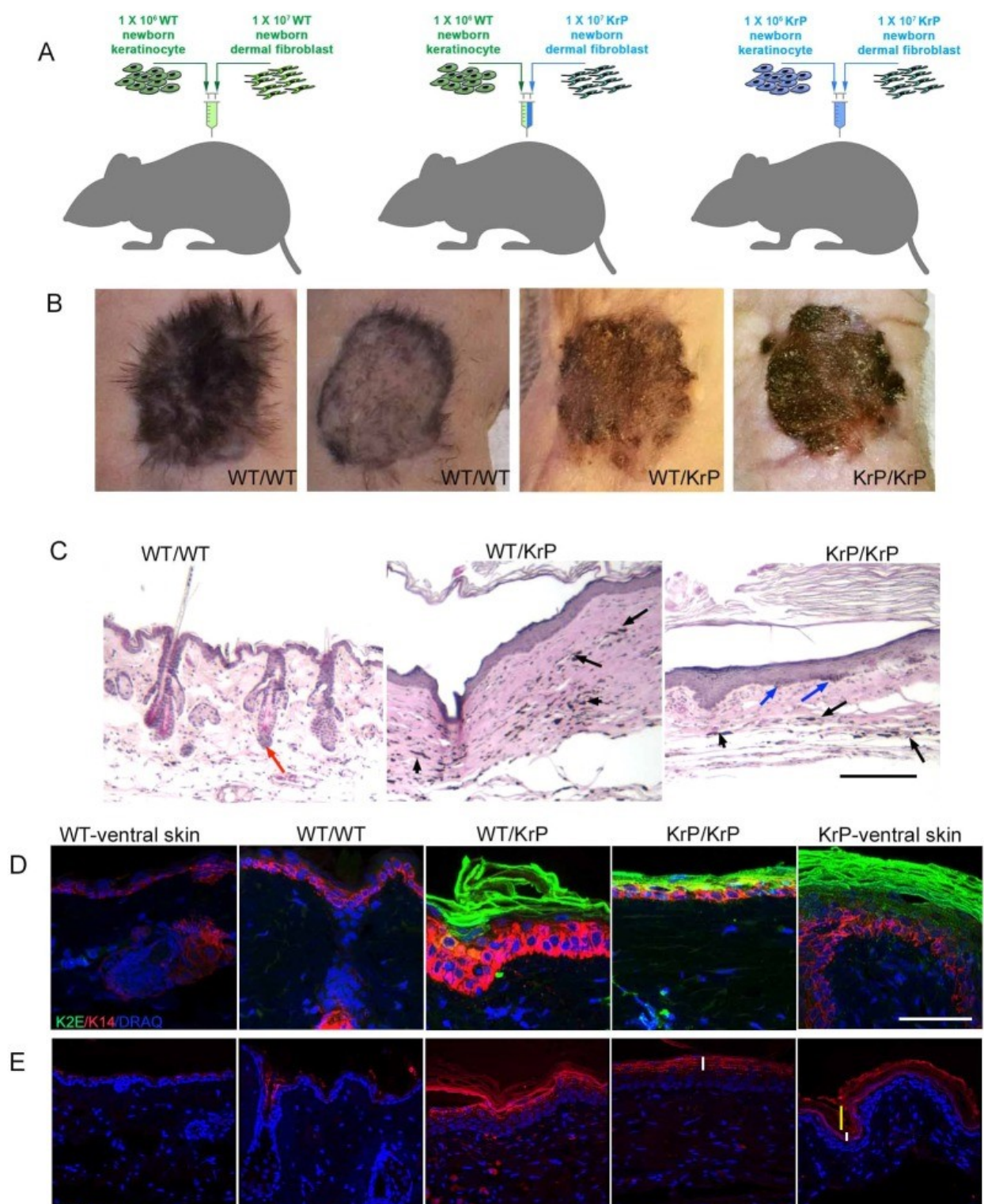
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Figures and Tables

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Fig. 1.

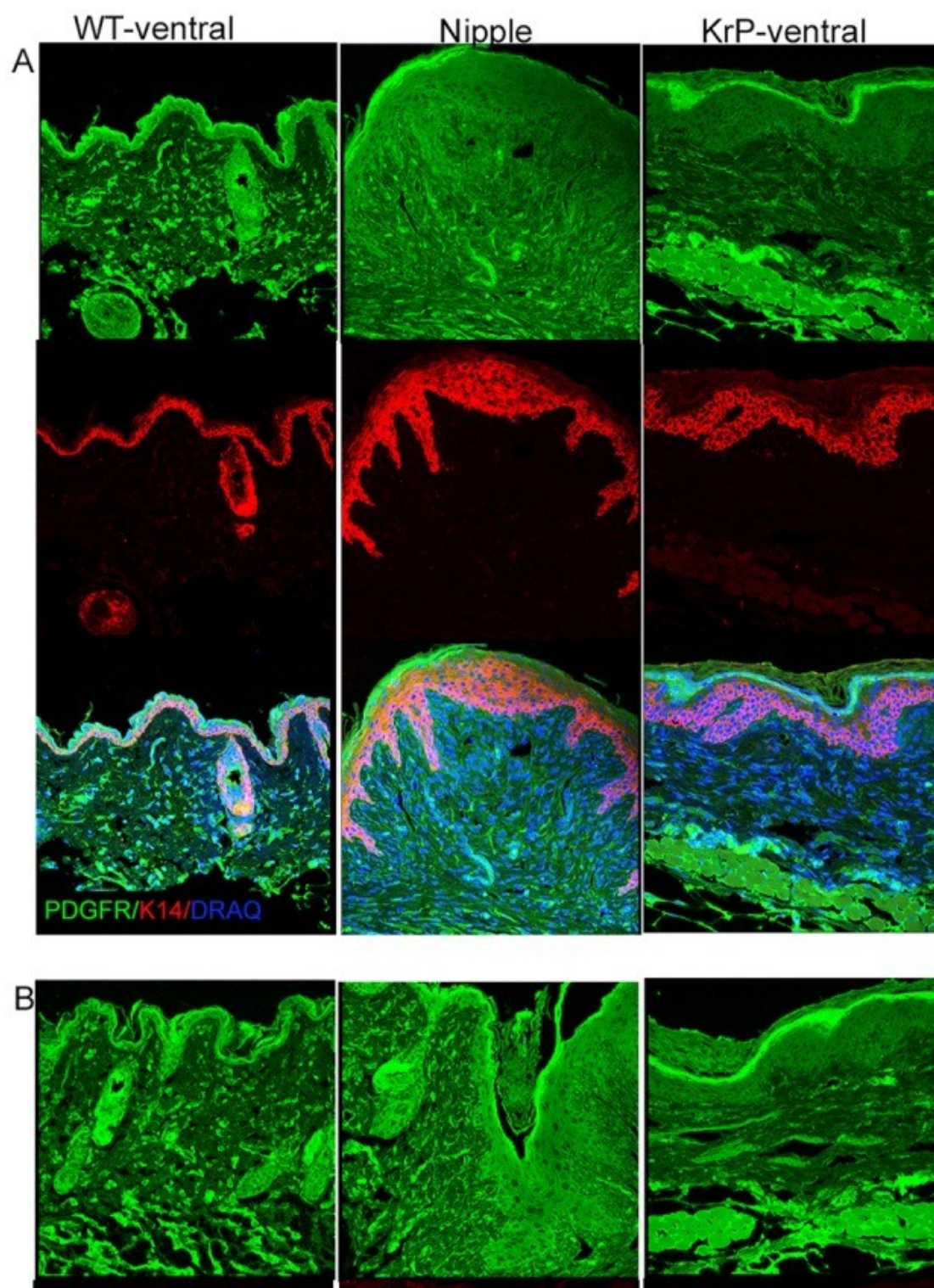


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Fibroblasts from nipple-like skin are sufficient to induce specialized epidermis. (A) Schematic outline of grafting experiment. (B) Hairless pigmented nipple-like skin was present in 3-month-old WT/KrP and KrP/KrP grafts on the dorsum of BALB/c *Foxn1*^{nu/nu} host mice. WT/WT grafts produced pigmented hair, but hair clipping revealed non-pigmented skin underneath. (C) H&E staining of grafts. Black arrows point at pigmented cells in the dermis of KrP/KrP and WT/KrP grafts. Occasionally pigment could be observed in the epidermis of the nipple-like grafts (blue arrows), whereas pigment was mainly confined to hair follicles in WT/WT grafts (red arrow). Scale bar: 400 μ m. (D) K2e and K14

immunostaining. WT/KrP and KrP/KrP grafts stain for K2e as does the KrP ventral skin. Scale bar: 50 μ m. (E) Filaggrin immunostaining. Multiple suprabasal layers in WT/KrP and KrP/KrP grafts and KrP ventral skin stained for filaggrin, compared to minimal labeling in the WT/WT grafts and intact WT skin. The white vertical bar indicates the granular layer and the yellow bar the cornified layer. Note the cornified layer is lost in the KrP/KrP graft due to antigen retrieval. Scale bar: 50 μ m. WT, wild type; KrP, *K14-PTHrP* transgenic mice; K2E, keratin 2e; K14, keratin 14; FIL, filaggrin; DRAQ, nuclei.

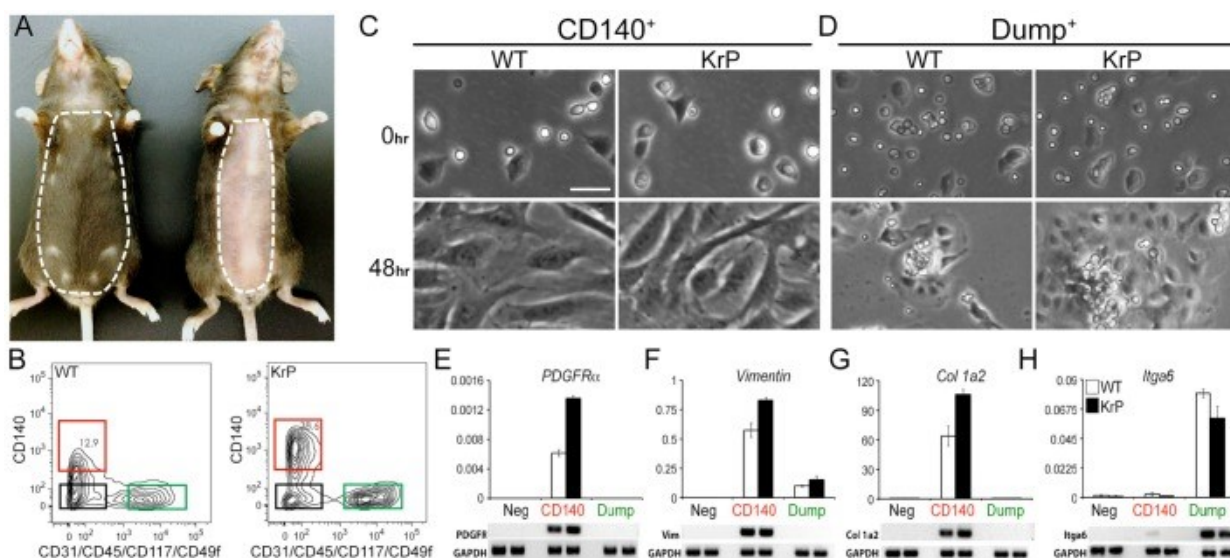
Fig. 2.



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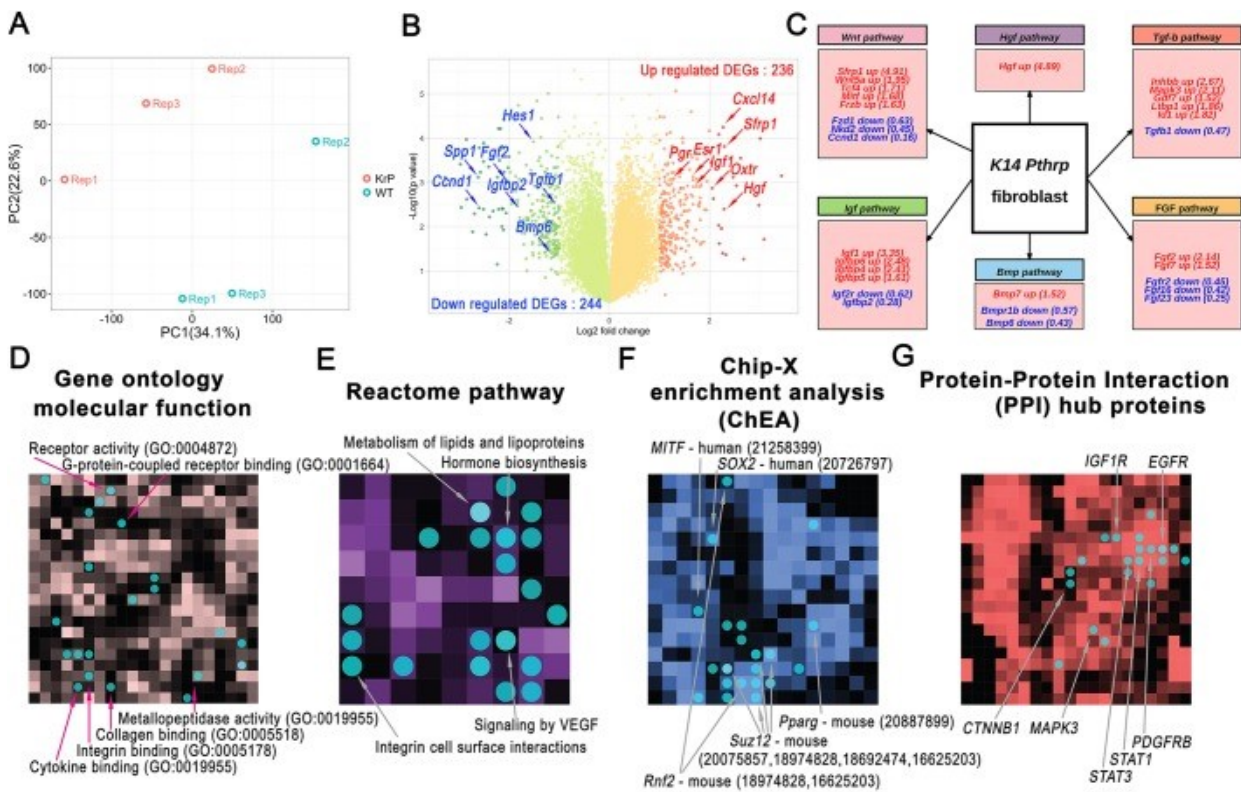
Pdgfra expression pattern in ventral skin and nipple tissue. Co-staining of ventral skin from WT mice (left), KrP mice (right) and WT virgin nipple (center) for *Pdgfra* (green), and (A) keratin 14 (K14, red), (B) vimentin (Vim, red) or (C) smooth muscle actin (SMA, red). Nuclei were counterstained blue with DRAQ5. Scale bar: 100 μ m. The different intensity for *Pdgfra* (PDGFR) staining among the sections reflects the use of different antibodies in A and B versus C. The intense labeling of the granular layer in the nipple and KrP samples shown in A and B is likely non-specific.

Fig. 3.



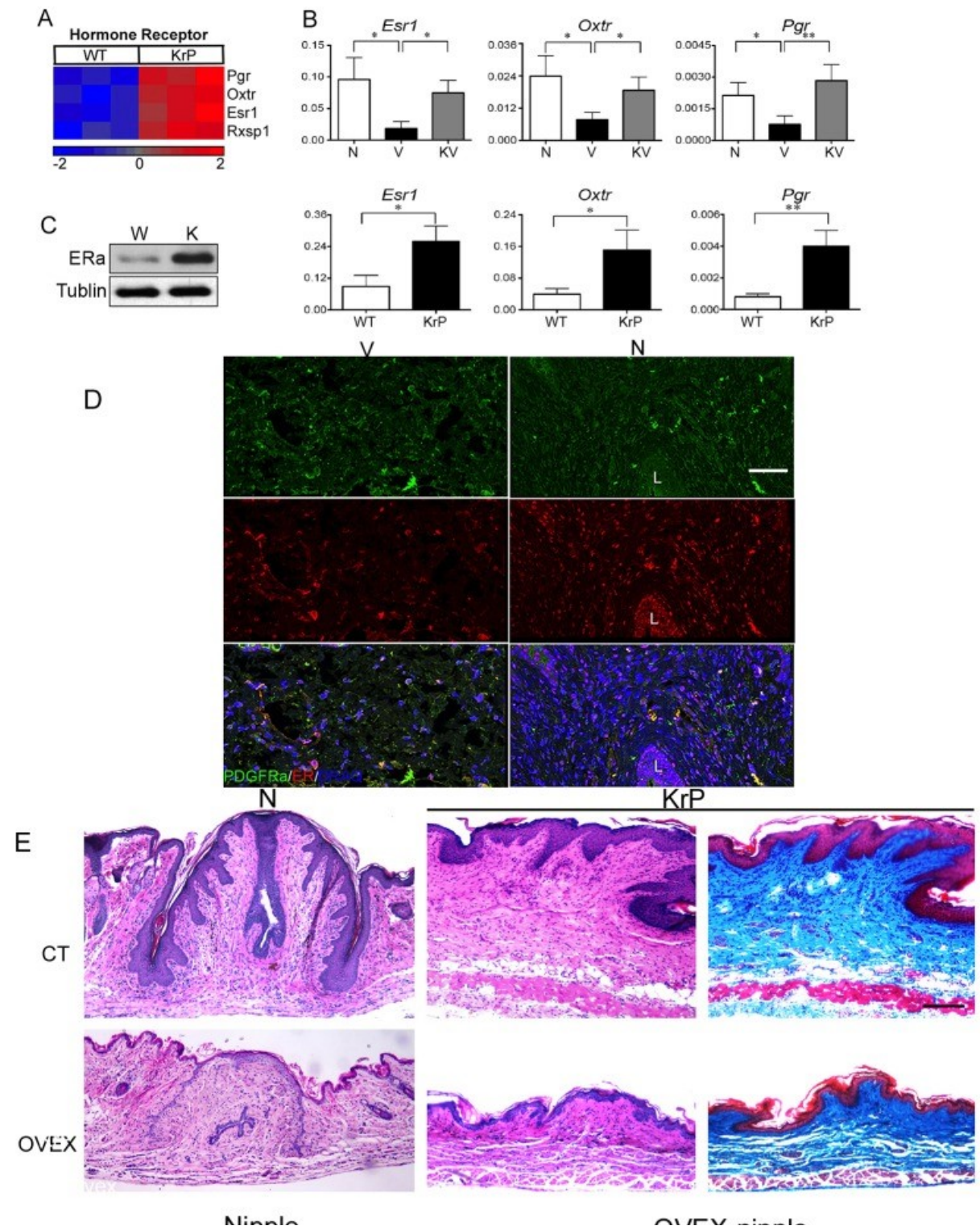
Pdgfra labels the mouse nipple fibroblast population. (A) The dotted outline marks the ventral skin in either WT (left) or KrP mice (right) used as the source for fibroblasts. (B) FACS gating strategy used to isolate Pdgfra⁺ cells (fibroblasts, red box), dump⁺ cells (mix of cells marked for CD31⁺, CD45⁺, CD117⁺ or CD49f⁺; green box) and double-negative Pdgfra⁻/Dump⁻ cells (black box). (C,D) Appearance of sorted Pdgfra⁺ cells (C) and dump-channel⁺ cells (D) immediately after sorting (0 hr) and after culture for 48 h. Only Pdgfra⁺ cells display fibroblast morphology after 48 h. Scale bar: 25 μ m. (E–H) qRT-PCR for select genes in sorted cell populations (CD140, Pdgfra⁺ cells). All target genes were normalized to *Gapdh*. Each bar represents the mean \pm s.d. of three independent experiments from five mice of each genotype. Bands resulting from qRT-PCR products visualized on 2% agarose gels stained with SYBR Green are shown underneath the graphs.

Fig. 4.



Bioinformatic comparison of KrP ventral fibroblasts and WT ventral fibroblasts. (A) Principal Component Analysis of KrP (red) versus WT ventral fibroblasts (blue) reveals global gene expression differences between cells of distinct genotypes. Dots represent individual biological replicates. (B) A volcano plot showing the relationship between gene expression fold changes and P -values across two genotypes. Red annotations mark differentially expressed genes (DEGs) upregulated in KrP ventral fibroblasts, while blue annotations mark downregulated DEGs. Positions for several up- and down-regulated DEGs on the volcano plot are annotated. (C) Summary of DEGs classified by their signaling pathway identity (WNT, HGF, TGF β , IGF, BMP or FGF). Red annotations identify DEGs upregulated in KrP ventral fibroblasts, while blue annotations identify downregulated DEGs. For each gene, the KrP versus WT fold-change is provided. (D-G) Network2Canvas analyses for four gene-set libraries: Gene Ontology molecular function (D), Reactome pathway (E), Chip-X enrichment analysis (ChEA) (F), and protein-protein interaction (PPI) hub proteins (G). Each canvas represents a specific gene-set library, where individual squares represent gene lists linked with gene-set library groups (or pathway gene groups). Square brightness is determined by its similarity to its eight neighbors. Each circle (blue) represents top 20 enriched pathways for the KrP versus WT DEGs within the specific gene-set library. Only relevant and statistically significant pathways are annotated here. Full lists of top 20 circles are in [Table S2](#).

Fig. 5.

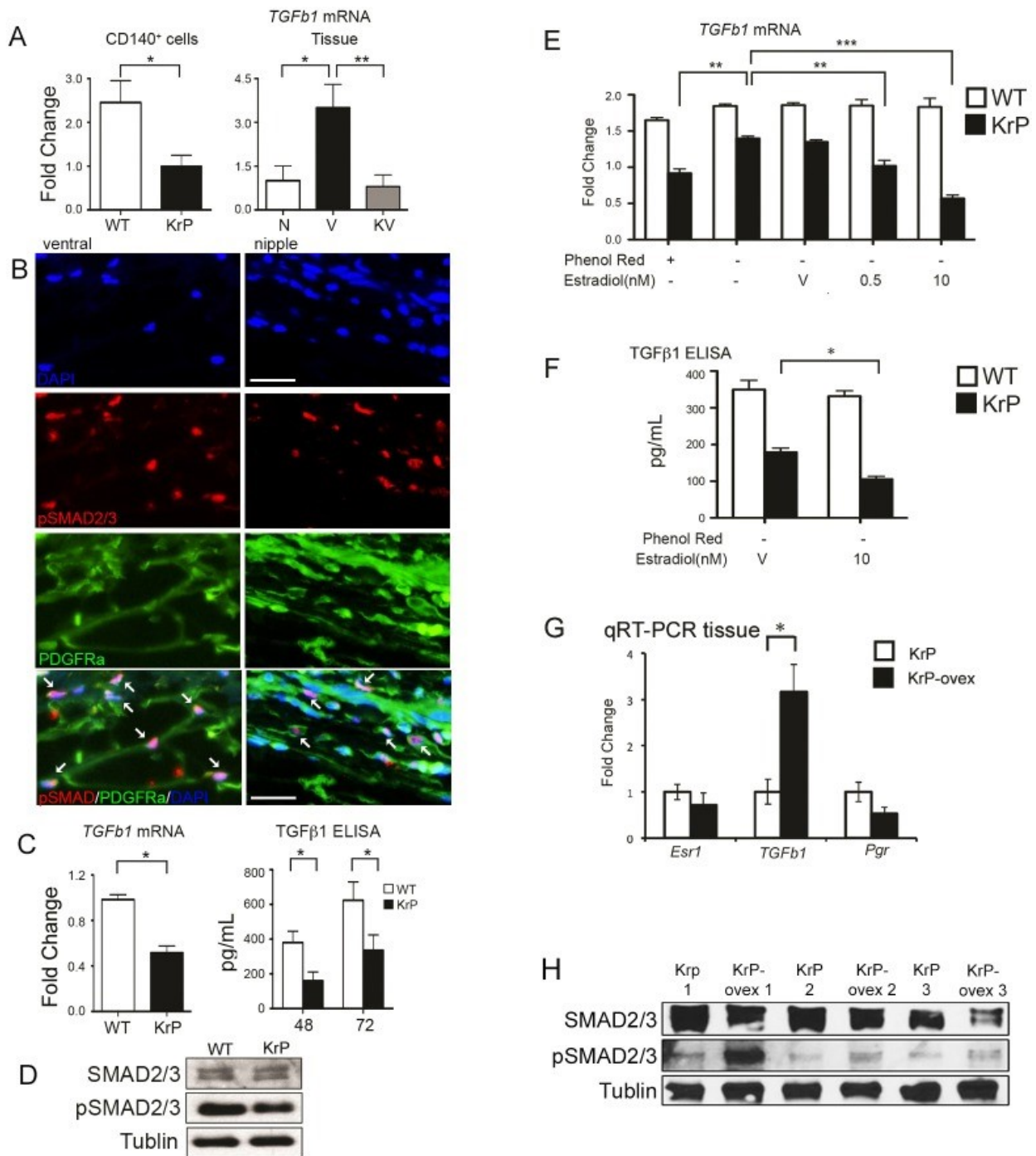


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Estrogen receptor expression and the role of ovarian hormones in the nipple. (A) Heatmap representations of hormone receptor expression in KrP versus WT fibroblasts in three independent experiments. (B) qRT-PCR for receptor gene levels in intact tissues and sorted fibroblasts. N, nipple of WT mouse; V, ventral skin of WT mouse; KV, ventral skin of KrP mouse; WT, sorted WT ventral fibroblast; KrP, sorted KrP fibroblasts. *Esr1*, estrogen receptor α ; *Oxt*, oxytocin receptor; *Pgr*, progesterone receptor. Expression was normalized to *Gapdh* and is expressed as the mean \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. (C) Western blot of cell extracts from cultured KrP and WT fibroblasts probed with anti-

estrogen receptor α (ER α) antibody using purified fibroblasts. The same protein extracts used in Fig. 6D were blotted in parallel and the same β -tubulin blot serves as loading control. W, WT ventral fibroblasts; K, KrP fibroblasts. (D) Co-expression patterns of Pdgfr α (green) and ER α (red) in virgin WT mice. Nuclei were counterstained blue with DRAQ5. V, ventral skin; N, nipple; L, lactiferous duct. Scale bar: 60 μ m. (E) Histology of WT nipples (N) and KrP ventral skin from ovexed mice. H&E and Masson's trichrome stains demonstrate thinner epidermis and increased size of collagen bundles under ovexed (OVEX) conditions. CT, control. Scale bar: 400 μ m. (F) Epidermal marker expression in nipples from ovexed and control mice. Layers of K2e, filaggrin and K14 labeled cells are markedly diminished under ovexed conditions. Scale bar: 50 μ m.

Fig. 6.

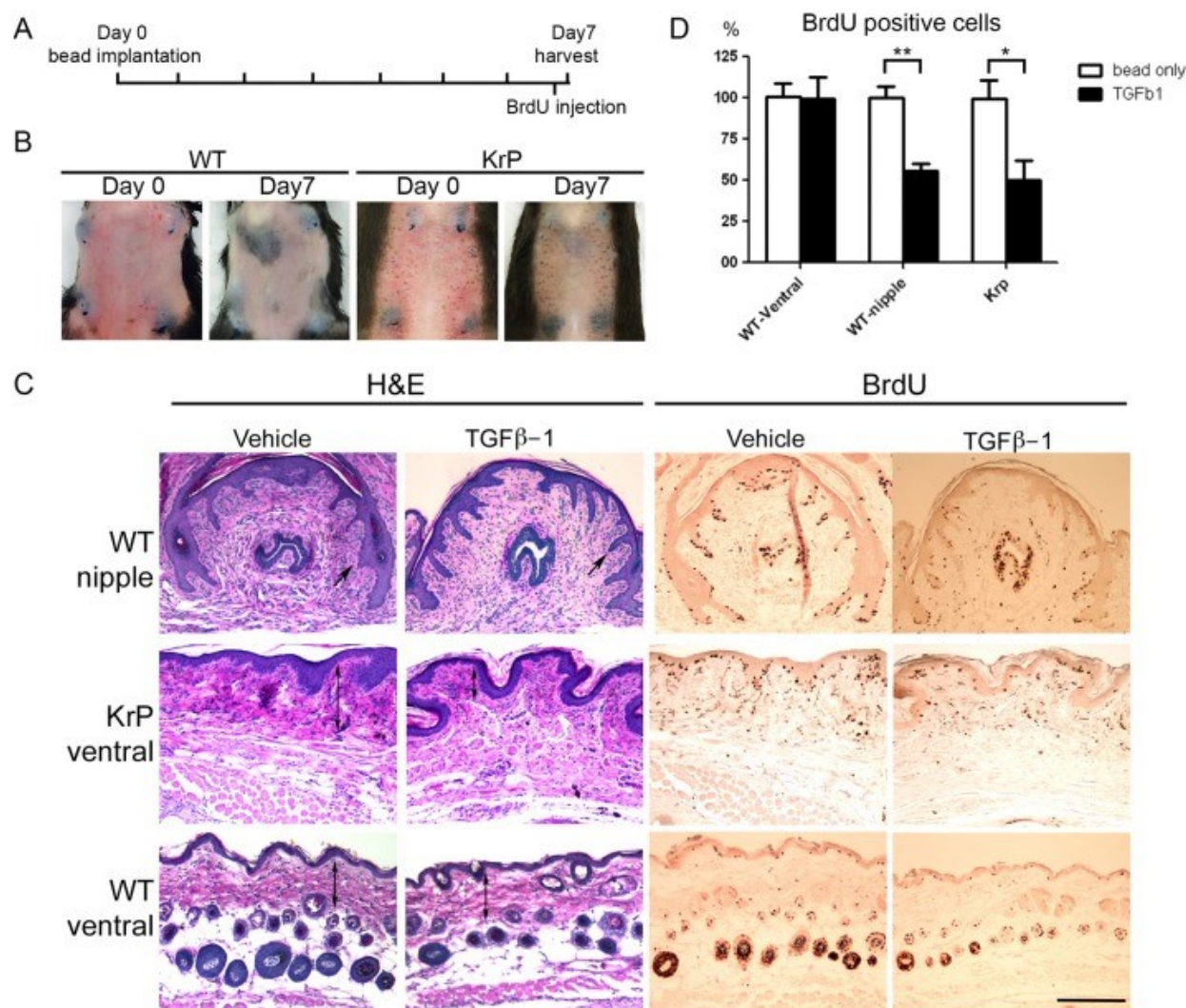


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Estrogen signaling negatively regulates TGF β signaling in nipple fibroblasts. (A) *Tgfb1* expression in CD140⁺ sorted fibroblasts and intact tissues. N, nipple of WT mouse; V, ventral skin of WT mouse; KV, ventral skin of KrP mouse. Expression was normalized to *Gapdh* and is expressed as the mean \pm s.d. of three independent experiments. (B) Co-staining for Pdgfr α (green) and pSmad2/3 (red) of ventral dermis and nipples of virgin WT mouse. Nuclei were counterstained with DAPI (blue). Arrows highlight co-expressing cells. Scale bars: 25 μ m. (C) *Tgfb1* expression levels in cultured fibroblasts (measured by qRT-PCR) and secreted levels of Tgf β 1 in culture medium of purified fibroblasts (measured by ELISA). Measurements were collected from three different cultures and performed in triplicate. (D) Western blot of purified fibroblasts probed with antibodies against Smad2/3 and pSmad2/3. The same protein extracts used in Fig. 5C were blotted in parallel and the same β -tubulin blot serves as loading control. (E) Relative *Tgfb1* levels in primary cultured fibroblasts with or without estradiol treatment. Expression of *Tgfb1* was measured by qRT-PCR and normalized to *Gapdh*. Each bar

represents the mean±s.d. of three independent experiments. (F) ELISA measurements of secreted Tgfb1 in culture medium harvested from primary cultured fibroblast with or without estradiol treatment. Each bar represents the mean±s.d. of triplicate cultures within a single experiment. The experiment was repeated twice with similar results. (G) Relative *Esr1*, *Tgfb1* and *Pgr* mRNA levels in ventral skin of ovexed as compared to age-matched control KrP mice. (H) Western blot of extracts from intact ventral tissues from ovexed and control KrP mice probed for Smad2/3 and pSmad2/3. β -tubulin was used as loading control. * P <0.05, ** P <0.01, *** P <0.001.

Fig. 7.



Sustained dermal activation of TGFβ signaling induces collagen remodeling and epidermal hypoplasia in the nipple. (A) Affi-gel blue beads with or without 0.2 μg of recombinant Tgfβ1 were injected intradermally into KrP ventral skin or WT nipple. Samples were collected at 7 days post-implantation. BrdU was injected 4 h prior to sample collection. (B) Affi-gel blue beads remain at the site of injection 7 days post implantation. (C) Bead-treated samples were stained for H&E and BrdU. Scale bar: 60 μm. (D) Quantification of the percentage of BrdU-positive keratinocytes relative to the length of basal epidermis. **P*<0.05, ***P*<0.01.

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