Supplemental Methods

Isolation of adult fibroblasts

The collagenase solution contained 1.25 mg/ml Collagenase type I (Gibco Thermo Fisher Scientific, Waltham, MA), 0.5 mg/ml Collagenase type II (Worthington Biochemical Co., Lakewood, NJ), 0.5 mg/ml Collagenase type IV (Sigma-Aldrich, St. Louis, MO), and 50 U/ml DNase I (Worthington Biochemical Co., Lakewood, NJ) in DMEM media at 37°C for one hour. The preparations were then passed through blunt-end needles several times to mechanically dissociate dermis before filtering the mixture through a 70 μm strainer.

Note that the ventral dermis of WT mice would contain a very small number of nipple fibroblasts.

Histology and immunofluorescence

Samples were collected, fixed and sectioned as previously described (Wu et al., 2015). The following primary antibodies against the following proteins were used: vimentin (mouse monoclonal, ab28028, Abcam, Cambridge MA), smooth muscle actin 1:50 (mouse monoclonal, A2547, Sigma-Aldrich, St. Louis MO), cytokeratin 2e 1:500 (mouse monoclonal 10R-C166a, Fitzgerald, Acton, MA), Keratin 14 (rabbit polyclonal, 905301, BioLegend, San Diego, CA), filaggrin 1:5,000 (rabbit polyclonal, 905801, BioLegend, San Diego, CA), Pdgfrα 1:200 (goat polyclonal, AF1062, R&D systems, Minneapolis MN and C-9 Santa Cruz Biotechnology, Dallas, TX), Esr1 (rabbit polyclonal, HC-20, Santa Cruz Biotechnology, Dallas, TX), Smad2/3 (rabbit polyclonal, FL-425, Santa Cruz Biotechnology, Dallas, TX),

pSmad2/3 1:200 (goat polyclonal, sc-11769, Santa Cruz Biotechnology, Dallas, TX). Secondary antibodies were used at 1:200 to 1:500 dilution (Jackson Immuno Research Laboratories Inc., West Grove, PA), including Alexa-Fluor 488 or 647- conjugated goat anti-rabbit or anti-mouse IgG. Nucleus staining was conducted using DAPI (D1306, Thermo Fisher Scientific, Waltham, MA) or Draq5 (62251, Thermo Fisher Scientific, Waltham, MA).

Western Blotting

The following primary antibodies were used: ERa (rabbit polyclonal, HC-20, Santa Cruz Biotechnology, Dallas, TX), Smad2/3 (rabbit polyclonal, FL-425, Santa Cruz Biotechnology, Dallas, TX), pSmad2/3 (sc-11769R, Santa Cruz Biotechnology, Dallas, TX). For the tissue analysis the following antibodies were used: β-tubulin 1:125 (mouse monoclonal, E7-s, Developmental Studies Hybridoma Bank, Iowa City IA), p-Smad2/3 (Ser 423/425) 1:100 (rabbit polyclonal, sc-11769, Santa Cruz Biotechnology, Dallas, TX), Smad2/3 (C-8) 1:200 (mouse monoclonal, sc-133098, Santa Cruz Biotechnology, Dallas, TX).

Supplementary Tables

Supplementary table S1. Microarray gene expression data and analysis. Data are generated from sorted Pdgfrα-positive ventral KrP and WT fibroblasts. Tab 1, raw gene expression data; tab 2, list of up-regulated genes; tab 3, list of down-regulated genes; tab 4, select signaling pathway genes; tab 5, reference signaling pathway gene list.

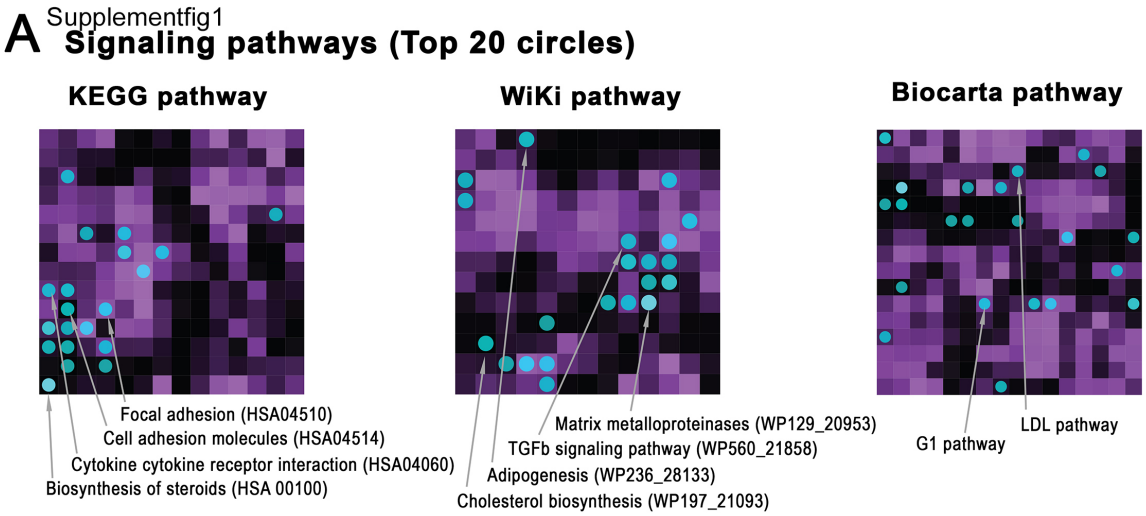
Click here to Download Table S1

Supplementary table S2. Differential gene expression analysis on Pdgfrα-positive ventral KrP and WT fibroblasts using Network2Canvas platform. Individual tabs contains lists of enriched terms within given gene networks.

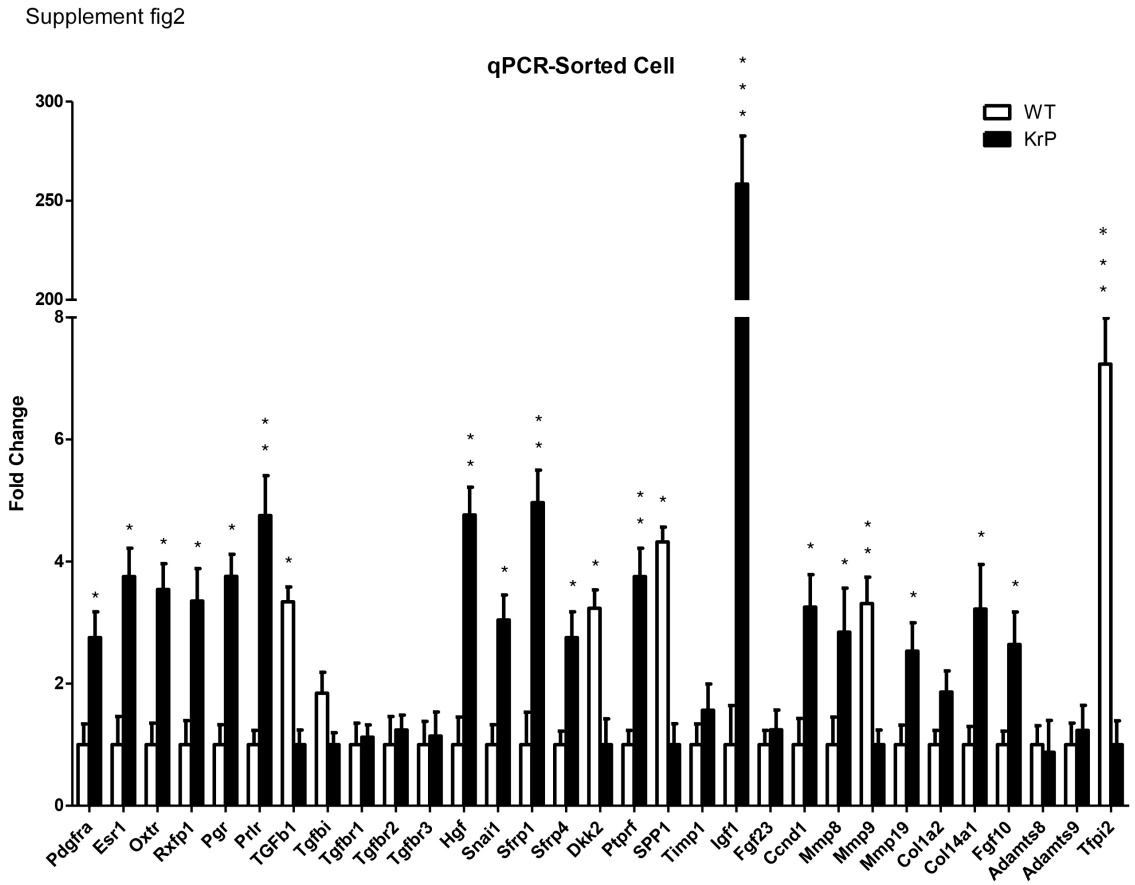
Click here to Download Table S2

Supplemental Figures

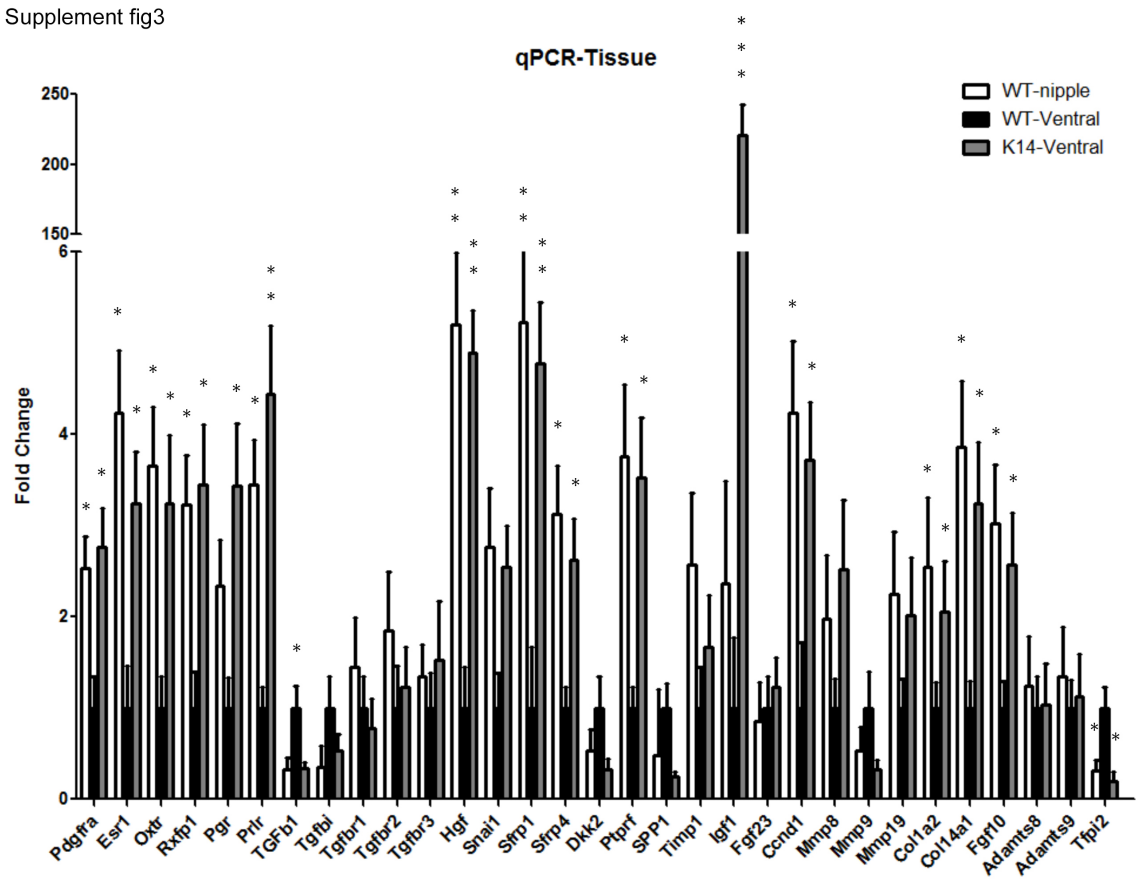
Supplementfig1



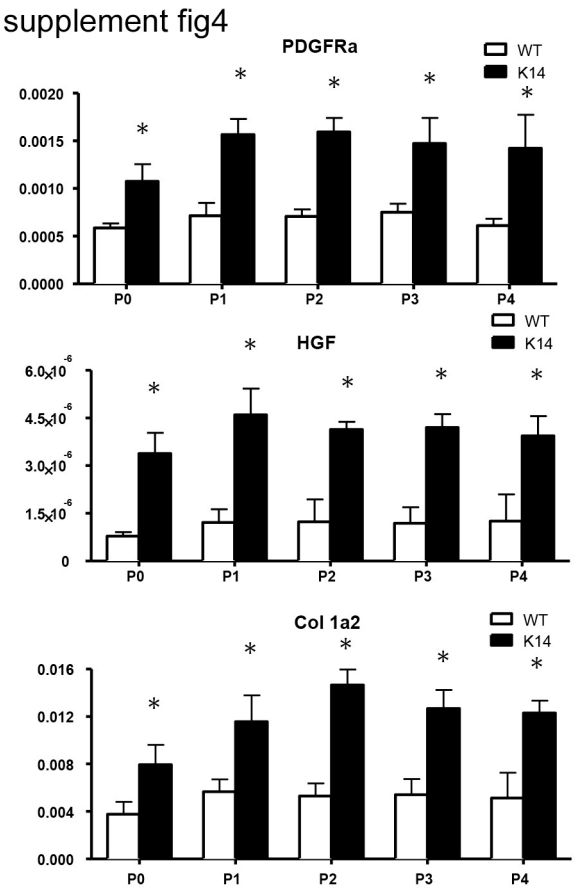
Supplemental figure S1. Network2Canvas analysis of KrP *vs.* WT ventral fibroblasts for seven gene-set libraries. Signaling pathways **(A),** Transcription **(B),** Kinase enrichment analysis **(C),** and Gene ontology and Cancer cell line encyclopedia **(D).** Each canvas represents a specific gene-set library, where each square represents a gene list linked with a gene-set library group. Square brightness is determined by its similarity to its eight neighbors. Each circle (blue) represents top 20 enriched pathways using KrP *vs.* WT DEGs within specific gene set library. Only relevant and statistically significant pathways are selectively annotated. Also see Supplementary tables S1 and S2.



Supplemental Figure S2. Confirmation of select differentially expressed genes by qRT-PCR using RNA from sorted cells. The differential expression of 30 transcripts (20 increased, 6 decreased and 4 unchanged in KrP relative to WT fibroblasts), many of which are part of the pathways identified by the Ingenuity and GO term analysis, was confirmed by qRT-PCR (Fig. S2). Of the transcripts evaluated, all with the exception of *Fgf23, Adamts9,* and *Timp1* exhibited substantial expression level differences. In addition, levels of *Ccd1* on qRT-PCR were opposite of the microarray data. Cells were sorted as in Figure 3**.** Relative mRNA levels of selected genes where the average lower expressing set of samples were arbitrarily set to 1. Each bar represents the average of three independent experiments (±s.d.). *P < 0.05, **P < 0.01, ***P < 0.001.

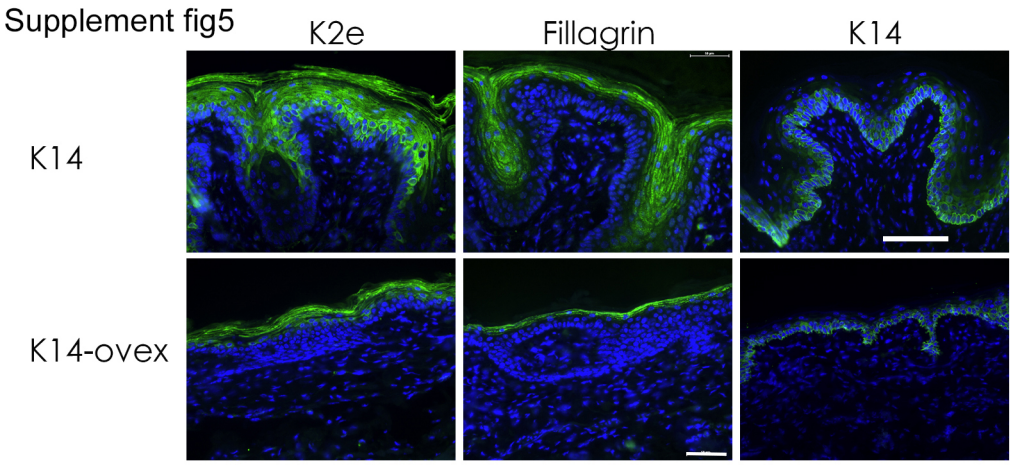


Supplemental Figure S3. Confirmation of select differentially expressed genes by qRT-PCR using RNA from intact tissues. To determine if the differential gene expression pattern of KrP fibroblasts was representative of the intact nipple, we validated it by qRT-PCR on RNA from the micro dissected nipples WT and KrP ventral skin. Indeed, all transcripts with the exception of the genes noted in figure 2 , were either elevated or reduced in the WT nipple as compared to WT non-nipple ventral skin in accordance with the KrP-to-WT fibroblasts differences (Fig. S3). In addition, *Igf1* expression was substantially lower (by ~100 fold) in the intact WT nipple as compared to KrP fibroblasts, or intact KrP ventral skin (Fig. S2, S3). Relative mRNA levels of selected genes where the WT ventral skin samples were arbitrarily set to 1. Each bar represents the average of three independent experiments (±s.d.). *P < 0.05, **P < 0.01, ***P < 0.001.

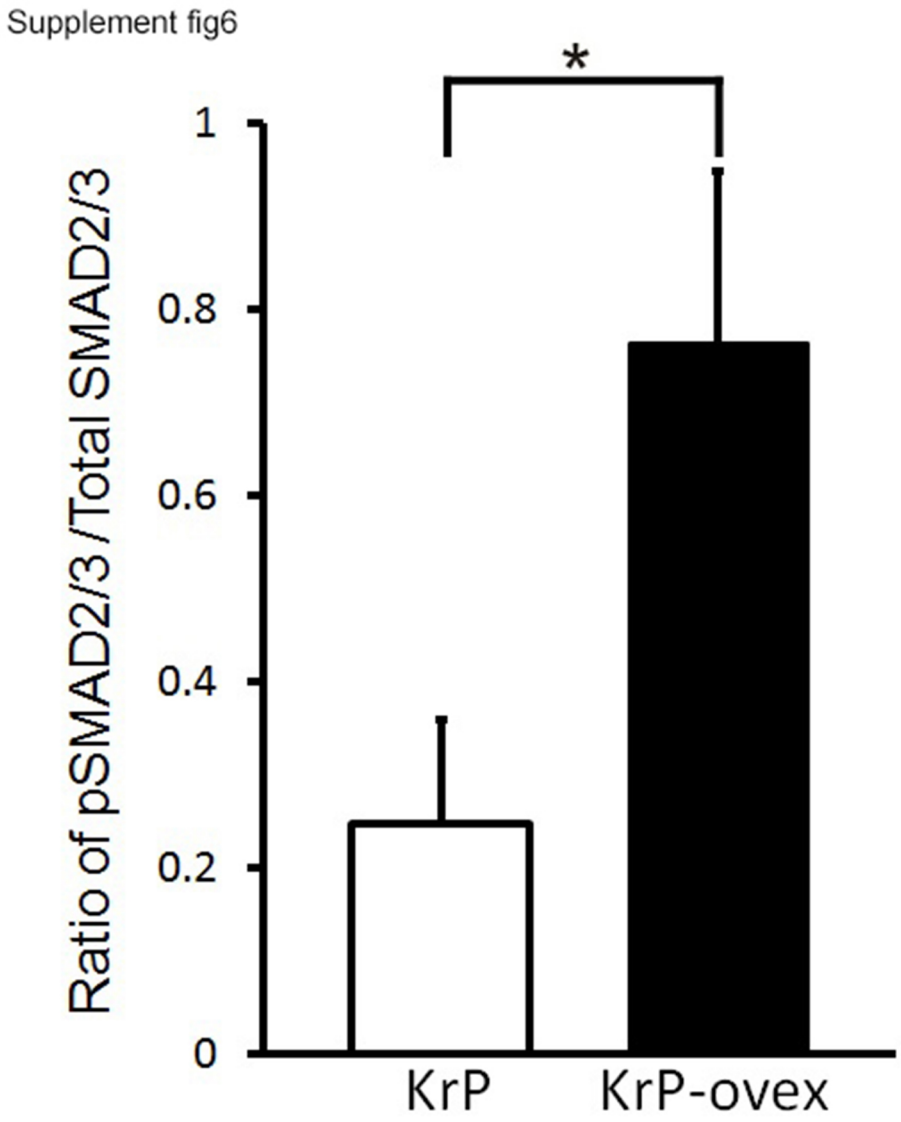


Supplemental Figure S4. Differential gene expression is maintained *in vitro*.

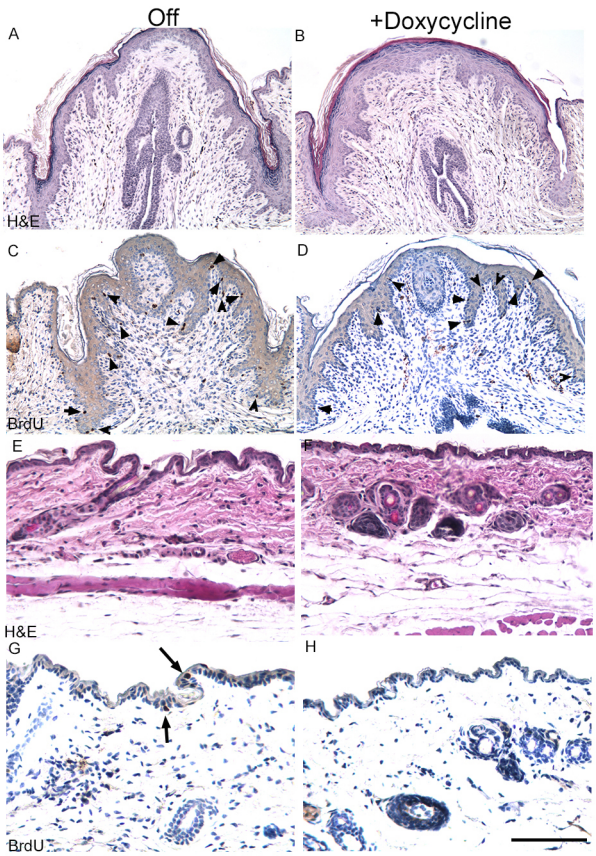
(A-D) To determine if the nipple fibroblast signature was stable in culture, we grew both WT and KrP ventral fibroblasts *in vitro* for up to 5 passages. We evaluated three signature transcripts (*Col1a*, *Pdgfrα* and *Hgf)* and found they remained differentially regulated in KrP relative to WT ventral fibroblasts, suggesting that some gene expression studies can be performed with cells grown *in vitro*. Relative mRNA levels in primary cultured fibroblasts at various passages. Expression of these transcripts was measured by qRT-PCR and normalized to *Gapdh* using absolute method. Each bar represents the average of three independent experiments (±s.d.). *P < 0.05.



Supplemental Figure S5. Differentiation marker expression in ovexed KrP skin. Samples are indicated on the left and antibody used on the top. Layers of K2e, filaggrin and K14 antibody labeled cells are markedly diminished in ovexed mice. . Scale bar: 50μm

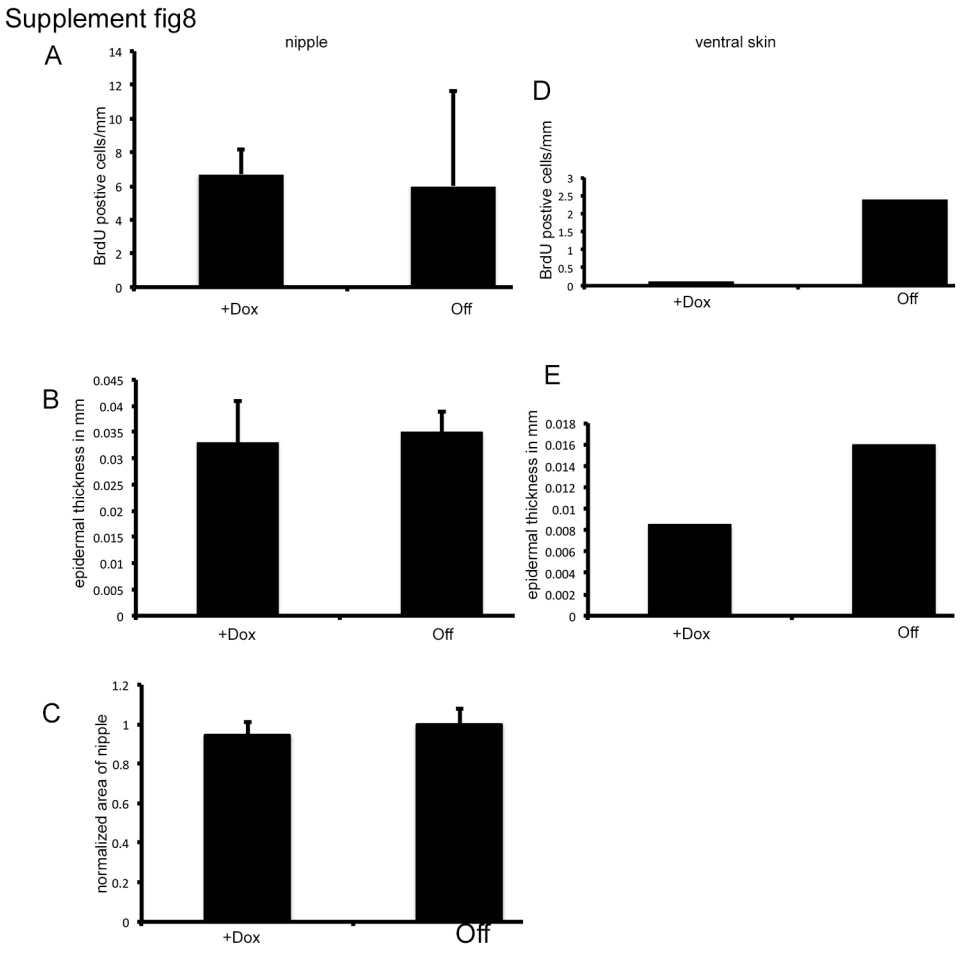


Supplemental Figure S6. Densitometry of pSmad2/3 *vs.* Smad2/3. Western blot shown in Fig. 6H was analyzed using imageJ to determine each band density. A ratio of pSmad2/3 *vs.* total Smad2/3 was calculated for KrP and ovexed KrP mice samples. Each bar represents the average ±s.d. of the triplicate samples blotted together. Extracts were evaluated two times in this manner with similar results. *P < 0.05.



Supplement fig7

Supplemental Figure S7. Overexpression of TGFβ1 in the epidermis. Six-week-old *K14-rTA/tetO-TGF β 1* virgin mice were either placed on doxycycline chow or control chow for 3-weeks, injected with BrdU and harvested. Number 4 and 5 nipples as well as non-nipple skin samples were processed for H&E and BrdU staining. **A, C, E** and **G** sections are from untreated controls and **B, D, F** and **H** from doxycycline treated animals. Nipple samples are **A-D** and ventral skin samples are **E-H.** Scale bar: 190 μm in A-D, 380 μm in E-H. Changes in the ventral epidermis of the TGFβ1 overexpressing mice were readily apparent in that portions of it pealed off during hair removal. Treatment of WT C57BL/6 mice with doxycycline chow failed to produce any detectable changes in the nipple or epidermis (not shown).



Supplemental Figure S8. BrdU analysis. Sections of eight nipples were analyzed using imageJ for **(A)** BrdU positive cells per epidermal/dermal junction length; **(B)** nipple epidermal thickness; **(C)** nipple area. No statistically significant differences in any of these parameters were observed between doxycycline treated nipples and those of controls. **(D)** BrdU positive cells per epidermal/dermal junction length and **(E)** epidermal thickness were evaluated for the ventral epidermis of the animals (n=2) and these parameters were markedly reduced in doxycycline treated animals.