Progesterone drives mammary secretory differentiation via RankL-mediated induction of Elf5 in luminal progenitor cells

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SUMMARY

Progesterone-RankL paracrine signaling has been proposed as a driver of stem cell expansion in the mammary gland, and Elf5 is essential for the differentiation of mammary epithelial progenitor cells. We demonstrate that Elf5 expression is induced by progesterone and that Elf5 and progesterone cooperate to promote alveolar development. The progesterone receptor and Elf5 are expressed in a mutually exclusive pattern, and we identify RankL as the paracrine mediator of the effects of progesterone on Elf5 expression in CD61+ progenitor cells and their consequent differentiation. Blockade of RankL action prevented progesterone-induced side branching and the expansion of Elf5+ mature luminal cells. These findings describe a mechanism by which steroid hormones can produce the expansion of steroid hormone receptor-negative mammary epithelial cells.

KEY WORDS: Elf5, Progesterone, RANKL, Mammary tissue, Mouse

INTRODUCTION

The mammary gland undergoes profound tissue remodeling during pregnancy in response to prolactin and progesterone (Pg), producing a massive elaboration of the milk-producing lobuloalveolar structures. Underlying these morphological changes is an epithelial cell hierarchy, headed by multipotent stem cells that undergo division and differentiation to produce lineage-committed progenitor cells, which in turn expand in number and differentiate into the mature epithelial cell types. Pg may regulate the proliferation of mammary stem cells via paracrine signaling from mature progesterone receptor (PR)-positive luminal cells (Asselin-Labat et al., 2010; Joshi et al., 2010), but it is not known how the resulting cellular flux is directed toward the secretory lineage. A clear candidate is the ets transcription factor Elf5.

Elf5 is expressed by mammary luminal progenitor cells and specifies alveolar cell fate (Oakes et al., 2008). Elf5 knockout produced failed alveolar morphogenesis and failed lactation (Oakes et al., 2008; Choi et al., 2009), sustained expression of characteristics of virgin epithelium during pregnancy, and decreased cellular proliferation. Conversely, forced expression of Elf5 in the nulliparous mammary epithelium caused precocious formation of alveoli and milk production (Oakes et al., 2008). During pregnancy, luminal progenitor cells accumulated in *Elf5*-null glands whereas forced expression of Elf5 in nulliparous mice resulted in erosion of this population (Oakes et al., 2008; Chakrabarti et al., 2012b). Tellingly, forcing the expression of Elf5 in prolactin receptor-null (PrlrKO) mammary epithelial cells rescued the failed alveolargenesis seen in PrlrKO mice (Harris et al., 2006). Thus, Elf5 is a key transcriptional effector of alveolargenesis, causing differentiation of progenitor cells towards the secretory lineage. Fixing cell fate decisions may be a generalized role of Elf5 (Lee and Ormandy, 2012). Given a similar lack of alveolar development during pregnancy in PRKO (Lydon et al., 1995) and PrlrKO (Ormandy et al., 1997) mice, it is possible that Pg and Prl may determine progenitor cell fate via Elf5 expression.

MATERIALS AND METHODS

Mice

All experiments involving mice were performed under the supervision of and in accordance with the regulations of the Garvan/St Vincent's Animal Experimentation Committee. Elf5/MTB transgenic mice (Oakes et al., 2008), Rank flox mice (Schramek et al., 2010) and MMTV-RankL mice (Fernandez-Valdivia et al., 2009) have been previously described.

Progesterone pellets (Pg; 5 mg/pellet, 21-day release; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously. Dox 700 mg/kg via feed-induced Elf5 expression. The anti RankL antibody (Oriental Yeast Company, Japan) and isotype control (Biolegend clone RTK2758) were injected intraperitoneally in saline at 10 mg/kg.

Quantitative PCR

Total RNA was extracted using TRIZOL Reagent (Invitrogen) and purified using RNeasy Mini Spin columns (QIAGEN). cDNA synthesis used Superscript II (Invitrogen). QPCR used TaqMan Gene Expression Assays and the Prism 7900HT Sequence Detection System were from Applied Biosystems.

Histology

Slides were blocked with 2.5% horse serum (Vector Laboratories, Burlingame, CA, USA) and, after milk antibody (Accurate Chemical and Scientific Corp NY) incubation, received Envision rabbit (Dako) secondary reagents for 30 minutes. SMA and Zo1 co-immunofluorescence antigen retrieval used pH 9 and 125°C for 10 seconds followed by Anti-Zo1 (1:100; Zymed Laboratories, San Francisco, CA, USA) and anti-SMA (1:500; Sigma-Aldrich) and secondary AlexaFluor 595-tagged anti-rabbit and AlexaFluor 488-tagged anti-mouse antibodies (1:500; Invitrogen). Elf5 and PR co-immunofluorescence used antigen retrieval: pH 9 at 125°C for 30 seconds then incubation with 1:100 rabbit anti-PR (Dako) and 1:300 goat anti-Elf5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C, followed by a 1-hour incubation with biotin-conjugated horse anti-goat secondary antibody (Vector Laboratories) then AlexaFluor 555 streptavidin conjugate and AlexaFluor 488-tagged donkey anti-rabbit (1:500; Invitrogen). ToPro-3 (1:200; Invitrogen) stained nuclei.

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Flow cytometry

Flow was performed as described previously (Shackleton et al., 2006; Asselin-Labat et al., 2007). Analysis used a BD Influx cell sorter (Becton Dickinson). Fluorochrome-conjugated antibodies were CD24-PE (BD Pharmingen, M1/69) and CD29-PE/Cy7 (Biolegend, clone HMβ1-1) and biotin-conjugated antibodies were CD61-APC (Invitrogen), CD31 (BD Pharmingen, clone390), CD45 (BD Pharmingen, clone30-F11) and Ter119 (BD Pharmingen). DAPI (100 ng/ml) marked dead cells. Analysis was performed using Flowjo v8.0.2 software (Tree Star).

RESULTS AND DISCUSSION
Progesterone induces Elf5 expression *in vivo*

Five mice per group were treated with Pg via subcutaneous slow-release hormone pellets or received a sham operation. An increase in the proportion of Elf5-positive cells (Fig. 1A,B, representative immunohistochemistry images of 10 random fields; higher magnification in 1C,D; and quantification in 1E) was seen. QPCR showed the same effect (Fig. 1F,G). Ovariectomized mice showed a similar fourfold increase in Elf5 after 7 days of Pg (data not shown). Elf5 is reduced in PR knockout mice (Fernandez-Valdivia et al., 2009) but loss of Elf5 does not affect PR expression (Oakes et al., 2008), indicating that Elf5 lies downstream of Pg signaling in the mammary gland.

Progesterone and Elf5 cooperate to promote alveolar development in the mammary gland

We treated *Elf5$^{+/-}$MTB$^{+/-}$* mice with doxycycline (Dox) to induce Elf5 expression (Oakes et al., 2008) with and without Pg treatment. Sham-operated animals without Dox treatment showed a ductal structure with primary ductal branching (Y-shaped branch pattern shown in Fig. 2A) but poorly developed secondary ductal branching (T-shaped branch pattern). Induction of Elf5 with Dox (Fig. 2B) caused the formation of clusters of alveoli at the ductal termini.

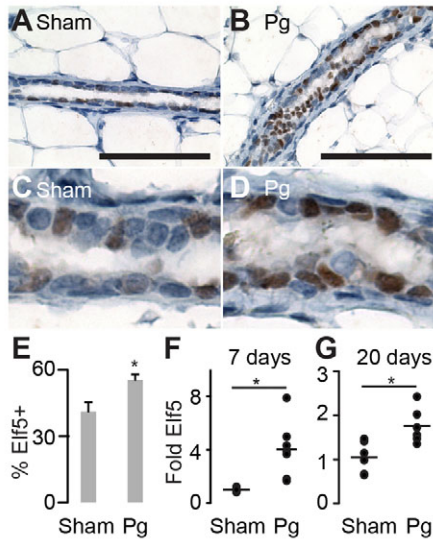Consistent with previous studies (Oakes et al., 2008), milk was detected in these structures, showing that cellular differentiation had occurred. Pg treatment (Fig. 2C) induced extensive secondary ductal side-branching. Animals treated with Pg and Dox showed the formation of extensive lobuloalveoli that exhibited a degree of mammary development far greater than that seen in the animals treated with progesterone or Dox alone (Fig. 2D). Staining for the luminal epithelial surface (Zo1) and basal epithelia (SMA) revealed that these lobuloalveoli contained clusters of alveoli with a polarized luminal epithelial layer (Fig. 2E). Lumens stained for milk (Fig. 2F, brown stain) and showed numerous lipid droplets, which are indicative of secretory initiation. This level of development
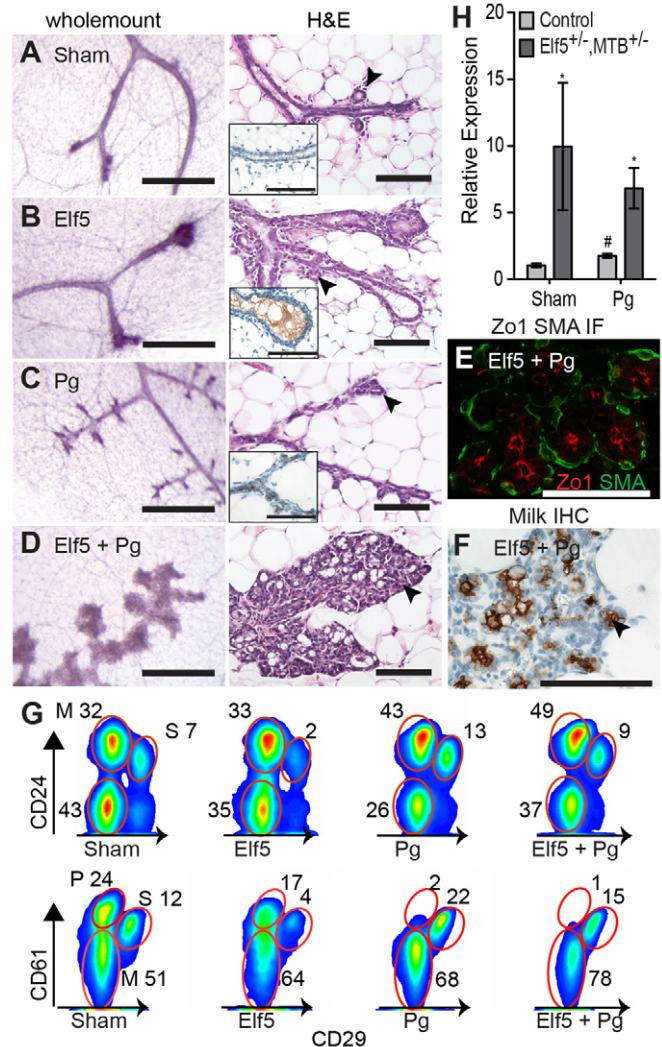

Fig. 1. Progesterone induces Elf5 expression *in vivo*. Mice were implanted with a subcutaneous Pg (5 mg/pellet, 21-day release) pellet or received a sham operation. (**A-D**) Elf5 immunohistochemistry staining in mammary glands collected 20 days after sham operation (A, low magnification; C, high magnification) or Pg pellet insertion (B,D). Scale bars: 100 μm. (**E**) Quantification of Elf5 immunohistochemistry staining presented as mean±s.e.m. for at least three animals. (**F,G**) RT-PCR analysis of Elf5 expression in mammary glands collected after 7 days (F) or 20 days (G) of Pg exposure. *P<0.05.

Fig. 2. Pg and Elf5 cooperate to promote alveolar development in the mammary gland. Doxycycline was administered to induce Elf5 expression in *Elf5$^{+/-}$ MTB$^{+/-}$* animals. (**A-D**) Carmine staining of mammary whole mounts and corresponding Hematoxylin and Eosin-stained sections with immunohistochemistry for milk shown in the insets, arrowheads indicate an alveolus. (**E**) Co-immunofluorescent staining of SMA (green, marker of myoepithelial cells located along the basement membrane) and Zo-1 (red, marker of the luminal alveolar surface). (**F**) Immunohistochemistry for milk protein expression from Pg- and Dox-treated mice. Scale bars: 100 μm. (**G**) Quantification of stem (S-CD29high/CD24high), progenitor (P-CD61 high) and mature cell (M-CD29low, CD61low) compartments by flow cytometry. (**H**) QPCR quantification of Elf5 expression levels, *P≤0.05, #P≤0.1 (t-test) for the comparison with corresponding control groups. Data are mean±s.e.m.

corresponds to that seen at mid-pregnancy in wild-type animals. These results establish a functional interaction between Pg and Elf5 to promote alveolar development well beyond that seen with either agent alone.

Flow cytometry was used to examine the stem and progenitor cell dynamics that underlie these developmental effects. Induction of Elf5 reduced the size of the CD61+ CD29 low progenitor cell compartment from 24% to 17% (Fig. 2G, lower panels labeled 'P'), as we have previously reported (Oakes et al., 2008). The relative size of the CD61+ CD29+ stem cell/myoepithelial cell compartment (labeled 'S') was also decreased, from 12% to 4%, consistent with a recent report using a conditional Elf5 knockout model (Chakrabarti et al., 2012b). Induction of Elf5 increased the proportion of mature luminal cells to 64%. By contrast, treatment with progesterone increased the proportion of cells in the stem cell-enriched myoepithelial compartment, as recently reported and associated with a large increase in stem cell numbers (Asselin-Labat et al., 2010; Gonzalez-Suarez et al., 2010; Joshi et al., 2010; Schramek et al., 2010). Progesterone greatly reduced the progenitor cell compartment, and increased the proportion of mature luminal cells. The combination of Pg and Elf5 tempered the progestin-induced expansion of the stem/myoepithelial compartment, returning it to near control levels, but totally ablated the progenitor cell compartment and led to the production of more mature luminal cells than either Elf5 or Pg alone (Fig. 2G). In these experiments, Dox treatment for 20 days increased Elf5 expression more than fivefold in both sham-operated and Pg-treated animals (Fig. 2H). Together, these data demonstrate that Pg and Elf5 force the differentiation of progenitor cells and that the combination of their actions is much more potent than their action alone.

RankL acts as a paracrine mediator of progesterone action to induce Elf5 in the progenitor cell population

Co-immunofluorescence for Elf5 and PR was performed on sections from virgin and pregnant mammary glands. Elf5 and PR showed a mutually exclusive pattern of expression throughout development (Fig. 3A,B). This pattern was also seen in mice treated with Pg (Fig. 3C,D) and in the Elf5 transgenic mouse (Fig. 3E,F). In virgin animals, Elf5 was expressed predominantly in luminal epithelial cells with a columnar shape, whereas PR was seen only in cells with

a round shape. In pregnant mammary glands the vast majority of luminal cells expressed Elf5 and were round; however, the few cells that continued to express PR did not express Elf5. This result implies that Pg must act via a paracrine mechanism to induce Elf5 expression.

RankL is a potential mediator of the paracrine effects of Pg on the stem cell compartment (Asselin-Labat et al., 2010; Joshi et al., 2010). Forced expression of RankL downstream of the MMTV promoter resulted in increased alveolar bud formation similar to that seen in Pg-treated Elf5 Tg mice (Fernandez-Valdivia et al., 2009). Elf5 transcripts were induced more than 150-fold by forced RankL expression (Fig. 3G). Conversely, Elf5 expression was decreased in mammary glands that lack the RankL receptor Rank (Fig. 3H). When mammary epithelial cells were sorted from estrogen- and progesterone-treated RankKO mammary glands, a large drop in Elf5 expression was seen in the CD61⁻ mature luminal cells and also in the CD61⁺ progenitor cells, consistent with an action of RankL in inducing Elf5 expression in the progenitor cell population, its major target population in the mammary gland (Fig. 3I). We investigated the potential for Elf5 to regulate Rank and RankL (Fig. 3J,K). We detected no significant changes in RankL expression in the mammary gland when Elf5 was induced, consistent with microarray studies in which RankL was unchanged in Elf5⁻/⁺ mammary glands (Oakes et al., 2008). We did observe a consistent and significant upregulation of the RankL receptor Rank, indicating the presence of a positive feedback regulatory loop between Elf5 and Rank that would serve to amplify the effects of RankL in Elf5-expressing cells.

Blockade of RankL signaling prevents the Pg induction of side branching and the expansion of Elf5-positive epithelial cells

We injected a RankL neutralizing antibody (xRankL) in the context of progesterone treatment (Fig. 4). Control animals, which received a sham operation and an isotype control antibody, displayed the typical mammary gland morphology and Elf5 expression pattern of virgin mice (Fig. 4A). Implantation of a progesterone pellet produced an increase in ductal side branching and expansion of Elf5⁺ cells (Fig. 4B, quantified in 4G). Mice receiving the xRankL in the absence of Pg showed no difference in ductal branching when compared with control mice (Fig. 4C), but when Pg-treated mice received xRankL,
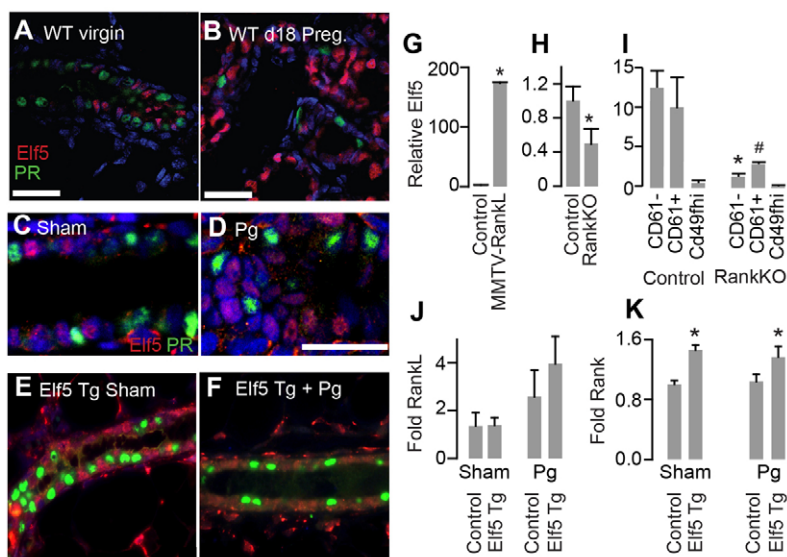

Fig. 3. Elf5 and PR are expressed in distinct cells within the mammary epithelium. (**A-F**) Co-immunofluorescent staining of PR (green) and Elf5 (red) in mammary sections from the indicated animals. ToPro-3 (blue) stains nuclei. Scale bars: 25 μm. (**G-I**) RT-PCR of Elf5 expression in mammary tissue collected from virgin MMTV-RANKL mice (G), RANK^Δmam mice (H) and from RANK^Δmam mice treated with Pg (I) and sorted by flow cytometry into the indicated populations. (**J,K**) Mice were treated with Pg (5 mg/pellet, 21-day release) pellet or received a sham operation. Doxycycline was administered to induce Elf5 in Elf5⁺/⁻,MTB⁺/⁻ animals. RT-PCR analysis of RankL (J) or Rank (K) expression. *P≤0.05, #P≤0.1 (t-test) for the comparison with corresponding control groups. Data are mean±s.e.m.
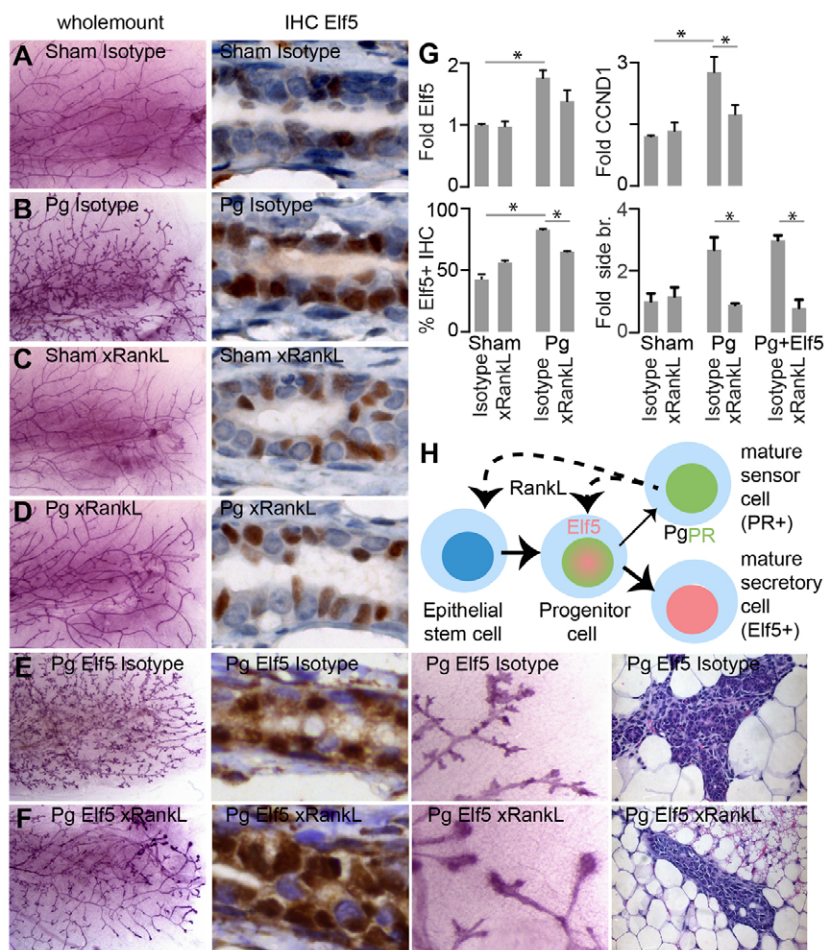
Fig. 4. Blockade of RankL action using a neutralizing antibody prevents ductal side branching and expansion of Elf5-positive epithelial cells. Mice were treated with Pg (5 mg/pellet, 21-day release) pellet or received a sham operation, and were treated with an anti RankL antibody for 7 days where indicated (xRankL) or isotype control (isotype). Elf5 was induced by Dox treatment as indicated (Elf5). (**A-F**) The effects on ductal side branching seen by whole-mount histology and associated changes in Elf5 staining by immunohistochemistry and Hematoxylin and Eosin for the indicated treatment groups. (**G**) Quantification of Elf5 positivity and expression, CCND1 expression and side branching relative to primary ducts. All data are presented as mean±s.e.m. for at least five animals. *$P<0.05$ (*t*-test) for the comparisons indicated by the bars. (**H**) Model of Elf5 action. Pg, which acts on PR+ sensor cells (Clarke, 2003) (green nuclei), causes them to secrete RankL. RankL (broken lines) then acts via a paracrine mechanism on both the stem cells (blue nuclei) and the progenitor cells (red/green nuclei). RankL acts on the stem cells to force their division and on progenitor cells, via induction of Elf5 expression, to force their differentiation towards the secretory cell fate.

ductal side branching did not occur, Pg induction of Cyclin D1 expression was abrogated and expansion of the Elf5+ cell population was inhibited (Fig. 4D,G). We induced Elf5 with Dox while treating with Pg and xRankL. Ductal side branching was completely suppressed by the antibody (Fig. 4G). The enhancement of alveolargenesis by Elf5 and Pg was also suppressed at mature ductal termini, but at immature termini, which are present at the periphery of the fat pad as terminal end buds, alveolargenesis occurred (Fig. 4E,F). We hypothesize that this differential response is due to the relative number of progenitor cells within each structure, as the flux of progenitor cells from stem cells is blocked by the antibody, but the differentiation of progenitor cells is promoted by Elf5 expression. These findings demonstrate that RankL mediates much of Pg action in the mammary gland, including ductal side branching and expansion of the Elf5+ cell population.

Pg and RankL have been implicated in regulation of the mammary cell hierarchy (Asselin-Labat et al., 2010; Gonzalez-Suarez et al., 2010; Joshi et al., 2010; Schramek et al., 2010), and we have previously demonstrated that Elf5 acts on luminal progenitor cells to specify the secretory cell lineage (Oakes et al., 2008). Our finding, that RankL can induce Elf5 expression, suggests that paracrine signals may influence luminal progenitor cells as well as stem cells. A simple model is shown in Fig. 4H. Without paracrine signaling to luminal progenitor cells, increased asymmetric division of the stem cells would cause an expansion in both secretory and sensor cell populations, rather than a preferential increase in secretory Elf5+ cells, which is observed (Fig. 3) and required for successful lactation.

Synthetic progestins used in hormone-replacement therapy may promote breast cancer (Travis and Key, 2003) and two studies have identified RankL as an important mediator (Gonzalez-Suarez et al., 2010; Schramek et al., 2010). In animals treated with the progestin MPA and the carcinogen DMBA, forced RankL expression promoted tumor formation, whereas loss of Rank retarded tumor formation. Thus, the Pg-RankL-Elf5 pathway may provide a mechanism that allows progestins to drive the early development of sex steroid hormone receptor-negative mammary cancers. RankL-mediated induction of Elf5 expression may have implications for progestin-driven breast cancer, as Elf5 has been shown to influence breast cancer phenotype (Kalyuga et al., 2012) and metastasis (Chakrabarti et al., 2012a).

Competing interests statement
The authors declare no competing financial interests.

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