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RESEARCH ARTICLE

In vivo reprogramming of non-mammary cells to an epithelial cell fate is independent of amphiregulin signaling

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ABSTRACT

Amphiregulin (AREG)$^{-/-}$ mice demonstrate impaired mammary development and form only rudimentary ductal epithelial trees; however, AREG$^{-/-}$ glands are still capable of undergoing alveologenesis and lactogenesis during pregnancy. Transplantation of AREG$^{-/-}$ mammary epithelial cells into cleared mouse mammary fat pads results in a diminished capacity for epithelial growth (~15%) as compared to that of wild-type mammary epithelial cells. To determine whether estrogen receptor α (ERα, also known as ESR1) and/or AREG signaling were necessary for non-mammary cell redirection, we inoculated either ERα$^{-/-}$ or AREG$^{-/-}$ mammary cells with non-mammary progenitor cells (WAP-Cre/Rosa26LacZ+ male testicular cells or GFP-positive embryonic neuronal stem cells). ERα$^{-/-}$ cells possessed a limited ability to grow or reprogram non-mammary cells in transplanted mammary fat pads. AREG$^{-/-}$ mammary cells were capable of redirecting both types of non-mammary cell populations to mammary phenotypes in regenerating mammary outgrowths. Transplantation of fragments from AREG-reprogrammed chimeric outgrowths resulted in secondary outgrowths in six out of ten fat pads, demonstrating the self-renewing capacity of the redirected non-mammary cells to contribute new progeny to chimeric outgrowths. Nestin was detected at the leading edges of developing alveoli, suggesting that its expression may be essential for lobular expansion.

KEY WORDS: Amphiregulin, Estrogen, Mammary, Stem cells, Reprogramming

INTRODUCTION

The mouse mammary gland microenvironment is a key determinant of mammary epithelial cell function and differentiation. Previously, we have demonstrated that this microenvironment acts as a niche that also controls the transdifferentiation of non-mammary stem or progenitor cells, including mouse testicular cells, neuronal stem cells and mesodermal cells, to a mammary cell fate (Booth et al., 2008; Bruno and Smith, 2012). Incorporation of these cells and their progeny into the mammary epithelium during *in vivo* regeneration demonstrates their ability to participate in normal mammary functions including ductal elongation, alveologenesis and milk secretion (Boulanger et al., 2007, 2012; Booth et al., 2008). These reprogrammed cells are maintained during serial transplantation studies, indicating that they have the ability to self-renew.

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Incorporation of either mouse or human breast cancer cells or human teratocarcinoma (Ntera-2) cells into the normal mammary niche attenuates their malignant phenotypes and promotes differentiation (Boulanger et al., 2013; Bussard et al., 2010; Booth et al., 2011). In all of these studies interaction with normal mammary epithelial cells (MECs) *in vivo* induced the transformation of non-mammary cells to a mammary epithelial cell fate. Our present study poses the question of whether growth-deficient mammary epithelial cells are able to perform the same task.

Mammary gland growth and differentiation largely occurs post puberty in mammals, including mice and humans, with epithelial proliferation and ductal expansion controlled by the cyclical production of mammary hormones including estrogen, progesterone and prolactin (Lyons et al., 1958; Nandi, 1958). Estrogen is arguably the most important in mammary gland development. Estrogen signaling in the mammary epithelium largely occurs via the estrogen receptor α (ERα; also known as ESR1) protein. Mice deficient for the ERα gene demonstrate a deficiency in post-pubertal ductal elongation and terminal end bud formation. However pre-pubertal growth is unaffected, as these mice contain a primitive epithelial rudiment (Korach et al., 1996; Boulanger et al., 2015; Mallepell et al., 2006). Thus, functional ERα signaling is absolutely required for the growth and differentiation of the mammary epithelium from puberty onwards.

Binding of estrogen and activation of ERα leads to transcription of numerous target genes including amphiregulin (AREG), a ligand for epidermal growth factor receptor (EGFR) (Peterson et al., 2015). AREG mediates estrogen-induced cell proliferation in the mammary epithelium and is required for post-pubertal mammary duct elongation (Ciarloni et al., 2007). AREG, a downstream target of both estrogen and progesterone signaling (Aupperlee et al., 2013), is also the primary growth factor induced by estradiol in pubertal mammary glands (Ciarloni et al., 2007) and is necessary for mammary end bud formation and ductal proliferation. AREG-knockout (AREG$^{-/-}$) mice demonstrate a severe deficiency in mammary gland growth post puberty; however, upon pregnancy, the mammary gland does undergo differentiation to form functional milk-producing lobules (Booth et al., 2010). Thus prior to pregnancy, AREG$^{-/-}$ mice mammary gland growth phenotypically mimics that seen in ERα$^{-/-}$ mice. It was shown previously (Ciarloni et al., 2007) that AREG$^{-/-}$ MECs mixed (1:10) with wild-type (WT) MECs proliferate and contribute to all compartments of a fully grown epithelial structure, indicating that AREG$^{-/-}$ epithelial cells are capable of full proliferation and differentiation in the presence of WT mammary epithelium *in vivo*.

Here, MECs from ERα$^{-/-}$ mice or AREG$^{-/-}$ mice were co-injected with WAP-Cre/Rosa26LacZ+ testicular cells (1:1) WAP (whey acidic protein) is promoter of Cre and is expressed only when induced by lactogenic hormones in mammary gland

mainly during late pregnancy and lactation into the cleared mammary fat pads of recipient mice to determine whether the combination of non-mammary cells and growth-restricted mammary cells could lead to normal mammary duct formation. Note that, whey acidic protein (WAP), which as used as the promoter to drive Cre, is expressed only when induced by lactogenic hormones in the mammary gland, mainly during late pregnancy and lactation. Similarly, AREG$^{-/-}$ MECs were also co-injected with constitutively expressing GFP-positive mouse neural stem cells (1:1). In all cases AREG$^{-/-}$ MECs supported the redirection of non-mammary cells to mammary epithelial cell fates. The outcomes are less dramatic with ER$\alpha^{-/-}$ cells mainly because the ER$\alpha^{-/-}$ cells consistently failed to grow when introduced into WT mammary fat pads even during pregnancy.

RESULTS

To determine whether ER$\alpha^{-/-}$ MECs can redirect WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells as WT MECs do, ER$\alpha^{-/-}$ cells were transplanted with or without WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells (1:1) into the cleared fourth inguinal fat pads of 3-week-old athymic Nu/Nu female mice. Recipient mice were mated and allowed to complete a full pregnancy in order to activate the WAP promoter, leading to Cre-mediated recombination and expression of the LacZ transgene from the constitutive Rosa26 promoter in male-derived testicular cells. Glands were harvested at least 10 days after forced weaning and gland growth, and LacZ expression was determined. Wild-type mammary cells were co-injected with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells as previously described and Lac-Z-positive cells could be identified mixed in with WT cells in the resulting outgrowths (Boulanger et al., 2007 and Fig. S1). As reported earlier (Mallepell et al., 2006), ER$\alpha^{-/-}$ cells injected alone formed only small rudimentary outgrowths in recipient fat pads (Fig. 1A). Ductal growth was not restored upon mixing with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells (Fig. 1B) whereas injection of ERα WT cells with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells led to complete filling of the recipient fat pad after a single pregnancy and involution (Fig. 1C). This finding indicates that MEC intrinsic ERα signaling is required for robust reprogramming of testicular cells. Small LacZ-positive outgrowths were obtained upon mixing testicular cells with ER$\alpha^{-/-}$ epithelium (Fig. 1B) following a single lactation and involution cycle; however, ductal structures were rudimentary and, upon pregnancy, no alveolar development was discernable (Table 1).

To assess whether AREG, a downstream target of estrogen signaling, could reprogram testicular cells, AREG-positive (WT) mammary cells or AREG$^{-/-}$ MECs were either injected on their own or co-injected with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells (1:1) into the cleared fourth inguinal fat pads of 3-week-old Nu/Nu female mice. Recipient mice were mated 4 weeks later and allowed to complete a full pregnancy. Glands were harvested at least 10 days after forced weaning, and gland growth, as well as LacZ expression, was determined.

AREG$^{-/-}$ cells injected alone phenotypically mimicked ER$\alpha^{-/-}$ cells, as only a small rudimentary growth occurred, even after prolonged (5 month) periods in the adult virgin fat pad (Fig. 1D). AREG$^{-/-}$ mammary cells co-injected with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells gave rise to chimeric outgrowths after a single pregnancy and lactation cycle, and these were capable of lobule development but only showed a slight ductal expansion; thus, chimeric outgrowths were unable to fill recipient fat pads (Fig. 1E). As shown previously, AREG-positive (WT) cells co-

injected with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells gave rise to full outgrowths after a single lactation and involution (Fig. 1F; Fig. S1; Boulanger et al., 2007). These findings are consistent with those that have previously shown that transplanted AREG$^{-/-}$ cells are able to produce lobule and minor ductal structures during pregnancy (Ciarloni et al., 2007). In four of six chimeric glands in which AREG$^{-/-}$ cells were mixed with WAP-Cre/Rosa26-lox-STOP-lox-LacZ+ testicular cells, slightly larger outgrowths were obtained post pregnancy. These outgrowths were positive for LacZ staining and PCR genotyping confirmed the presence of the male-specific *Sry* gene and the AREG genotypes (Fig. 1G). All of the positive outgrowths displayed a very slight increase in ductal length, but no terminal end buds (TEBs) (Table 2). No growth was observed in two of the six inoculated fat pads described above.

To determine whether AREG$^{-/-}$ MECs could redirect other non-mammary cell populations, constitutively GFP-positive mouse neuronal stem cells were mixed 1:1 with AREG$^{-/-}$ mammary cells and injected into recipient fat pads. In this case, no pregnancy was required to activate the reporter, which was expressed from a ubiquitous promoter. AREG$^{-/-}$ cells were able to redirect GFP-positive neuronal cells to mammary cells in both virgin (Fig. 2A) and parous (Fig. 2B, Table 2, items highlighted with an asterisk) mice (n=2 of 4, and 4 of 6, respectively). Whole mounts of chimeric glands showed limited ductal growth of GFP-positive in virgin animals and GFP-positive larger acinar structures in pregnant hosts. Cross sections of outgrowths were stained with DAPI to identify AREG$^{-/-}$ portions of the chimeric outgrowths in virgin (Fig. 2C) and parous (Fig. 2D) glands, in which all structures were associated with GFP-positive cells. Further, whole-mounted glands were also stained with Carmine Alum to determine the extent of AREG$^{-/-}$ growth (Fig. 2E). Mammary outgrowths were confined only to areas of the fat pads positive for GFP expression, and little to no growth of ductal structures was observed in AREG$^{-/-}$ cells that were GFP negative. Secondary transplantation of GFP-positive chimeric outgrowth fragments to Nu/Nu hosts yielded positive growth in seven of ten virgin fat pads, with larger epithelial growth than initial generations (Table 2), thus demonstrating the self-renewing capacity of the reprogrammed cells. Therefore, equivalent to our findings using male testicular cells, AREG$^{-/-}$ mammary cells were able to redirect neuronal stem cells to a mammary cell fate; however, the growth-deficient phenotype of the AREG$^{-/-}$ cells could not be rescued by the redirected GFP-positive neuronal stem cells in chimeric outgrowths.

GFP-positive chimeric outgrowths were then sectioned and stained for both mammary cell and neuronal cell markers. Consistent with their morphological appearance of normal mammary epithelium, redirected GFP-positive epithelial structures expressed ERα in virgin hosts (Fig. 3A) and, upon pregnancy, produced and secreted the milk protein casein (Fig. 3B). Estrogen-receptor-positive cells were composed of both GFP-positive reprogrammed cells (yellow arrow, Fig. 3A) and GFP-negative AREG$^{-/-}$ mammary cells (white arrow, Fig. 3A). Stained sections contained smooth muscle actin (SMA)-positive cells; however SMA-positive cells do not appear to be positive for GFP, suggesting that the AREG$^{-/-}$ MECs made up the predominate SMA-positive population (white arrows, Fig. 3C). Chimeric glands were also positive for progesterone receptor (Fig. 3D), which similar to ERα staining, was made up of both GFP-positive reprogrammed cells (yellow arrows) and GFP-negative mammary cells (white arrows). Consistent with the neuronal–AREG$^{-/-}$ chimeric outgrowths, chimeric outgrowths between WAP-Cre-Rosa26-lox-STOP-lox-
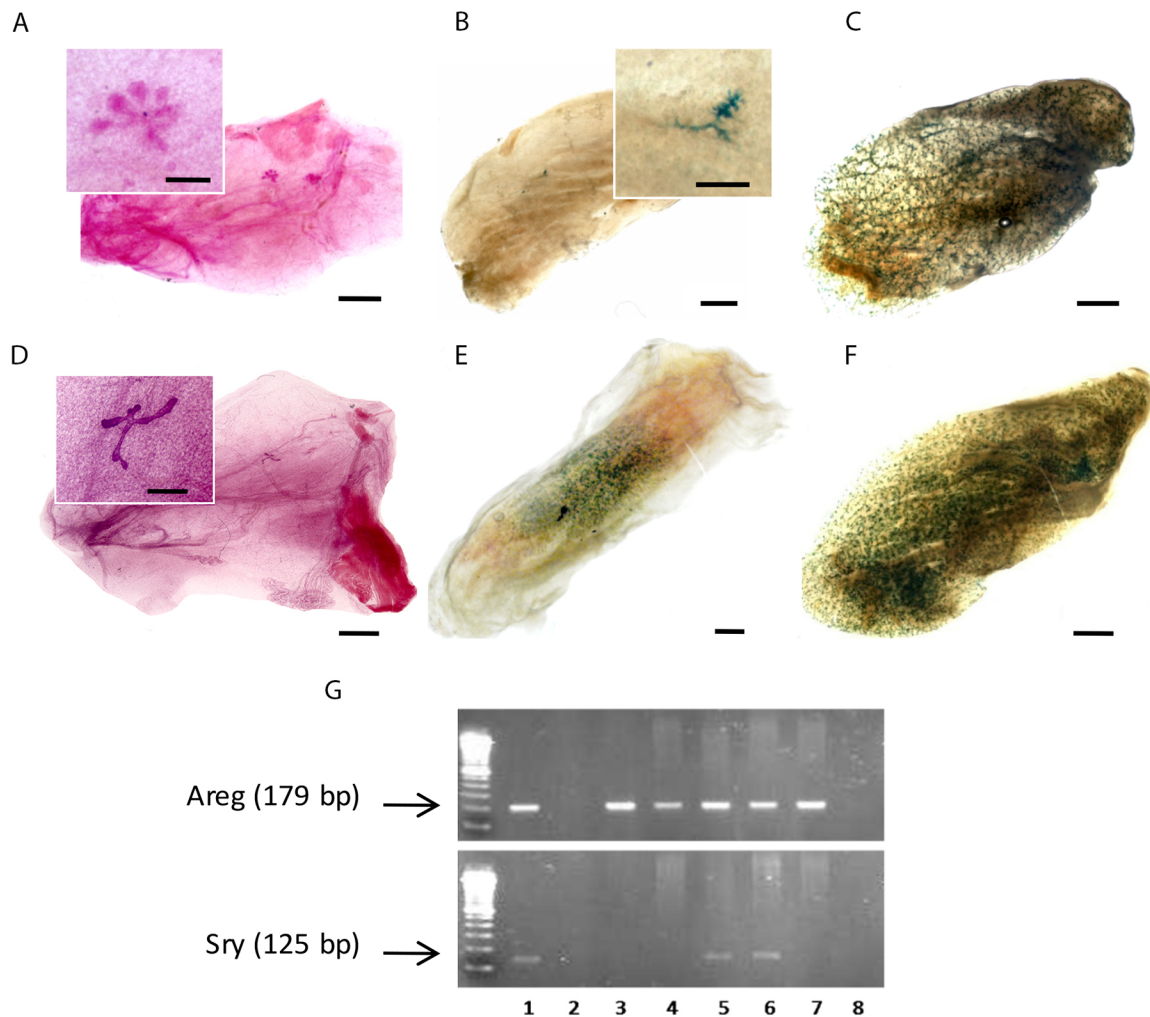
Fig. 1. Outgrowth and reprogramming potential of ERα$^{-/-}$ and AREG$^{-/-}$ mammary epithelial cells. Outgrowths from ERα$^{-/-}$ MECs transplanted alone, stained with Carmine Alum (A), or with LacZ-positive testicular cells, stained with X-gal (B). Both form only rudimentary structures. ERα-positive mammary cells form full chimeric outgrowths when mixed with WAP-Cre-activated testicular cells following a single lactation–involution cycle (C). Outgrowths from AREG$^{-/-}$ mammary cells alone, stained with Carmine Alum (D), generate only partial outgrowths with incomplete ductal elongation similar to those formed by ERα$^{-/-}$ cells. Addition of WAP-Cre-activated LacZ-positive testicular cells allows chimerism after a single lactation–involution cycle, but does not dramatically improve ductal elongation (E). AREG-positive mammary cells form full chimeric outgrowths when mixed with WAP-Cre-activated testicular cells after lactation and involution (F). Scale bars: 1 mm, insets 200 μm. (G) Male DNA (*Sry* gene) is detectable in chimeric outgrowths. Lane 1, male mouse tail DNA; lane 2, AREG$^{-/-}$ MECs; lane 3, AREG$^{+/+}$ MECs, lane 4, AREG$^{-/-}$ MEC outgrowth (WT fat pad); lane 5, AREG$^{-/-}$ and LacZ-positive testicular cells; lane 6, AREG-positive and LacZ-positive testicular cells; lane 7, #3 gland from host mouse, lane 8, water. Staining images are representative of two glands per group, with staining performed in triplicate; total numbers of replicates are shown in Tables 1 and 2.

LacZ+ testicular cells and AREG$^{-/-}$ cells also contained SMA-positive LacZ-negative cells (Fig. 3E); however, progesterone receptor-positive cells were not LacZ-positive indicating testicular cells did not express progesterone receptor in the reprogrammed glands, in contrast to GFP-positive neuronal cells. Our immunological staining demonstrates that reprogrammed neural stem cells express phenotypic markers including the mammary hormone receptors ERα and progesterone receptor. In addition, they also produce and secrete milk proteins, which demonstrates their complete acquisition of mammary cell function.

Previously, we demonstrated that nestin-positive neural stem cells could still be isolated after chimeric mammary growth (Booth et al., 2008). Other investigators have reported nestin staining in the basal cells of epithelium in intact mouse mammary glands (Li et al., 2007). To determine whether reprogrammed GFP-positive cells mixed with AREG$^{-/-}$ mammary cells still expressed the neural stem cell marker nestin, we stained outgrowths for nestin expression and, surprisingly, while small nestin-positive (red) cells could be found along the periphery of lobular structures (Fig. S2A,D), these cells were negative for GFP expression suggesting that they arise from a non-neuronal source. As nestin has also been used as a marker of basal mammary stem cells, we co-stained sections for nestin and SMA (purple). We found that while positive cells for both were found in the basal layer, they were not co-expressed by the same cells (Fig. S2B,C). However, nestin-positive cells were largely found in pregnant mammary tissues whereas little to no positive staining was observed in virgin outgrowths (data not shown). To confirm that nestin-positive cells localized to lobular structures, hyperplastic alveolar mammary outgrowths from Czech mice were stained for nestin and SMA (Fig. S2D). Similar to the chimeric results, nestin-positive cells were observed at the expanding edges of acinar structures (white arrows), whereas singly positive SMA

Table 1. ERα and WAP-Cre/Rosa26LacZ+ testicular cells chimera experiments

Mammary cells (×10³)	Testicular cells (×10³)	Outgrowth
50 ERα⁺/⁺	–	+++
50 ERα⁺/⁺	–	+++
50 ERα⁺/⁺	–	+++
50 ERα⁺/⁺	50 WCR26/LacZ⁺	+++
50 ERα⁻/⁻	–	+
50 ERα⁻/⁻	–	–
50 ERα⁻/⁻	–	–
50 ERα⁻/⁻	–	–
50 ERα⁻/⁻	50 WCR26/LacZ⁺	+
50 ERα⁻/⁻	50 WCR26/LacZ⁺	+
50 ERα⁻/⁻	50 WCR26/LacZ⁺	+
50 ERα⁻/⁻	50 WCR26/LacZ⁺	–
50 ERα⁻/⁻	50 WCR26/LacZ⁺	–
50 ERα⁻/⁻	50 WCR26/LacZ⁺	–

Results of transplantation of ERα⁺/⁺ and ERα⁻/⁻ mammary cells mixed with WAP-Cre/Rosa26LacZ+ (WCR26/LacZ⁺) testicular cells. The host strain was athymic Nu/Nu. Outgrowth: –, none; +, rudimentary; (<10% fat pad filled); ++, low (10–50% fat pad filled); +++, substantial (>50% fat pad filled).

Table 2. AREG and non-mammary stem cell chimera experiments

Mammary cells (×10³)	Testicular cells (×10³)	Neural stem cells (×10³)	Outgrowth
50 AREG⁺/⁺	–	–	+++
50 AREG⁺/⁺	–	–	+++
50 AREG⁺/⁺	100 WCR26/LacZ⁺	–	+++*
50 AREG⁺/⁺	100 WCR26/LacZ⁺	–	+++*
50 AREG⁺/⁺	–	50 NSC	+++
50 AREG⁺/⁺	–	50 NSC	+++
–	–	100 NSC	–
–	–	100 NSC	–
–	–	100 NSC	–
–	–	100 NSC	–
50 AREG⁻/⁻	–	–	–*
50 AREG⁻/⁻	–	–	+*
50 AREG⁻/⁻	–	–	++
50 AREG⁻/⁻	–	–	++
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	++*
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	++*
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	++*
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	+*
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	–*
50 AREG⁻/⁻	100 WCR26/LacZ⁺	–	–*
50 AREG⁻/⁻	–	50 NSC	+*
50 AREG⁻/⁻	–	50 NSC	+*
50 AREG⁻/⁻	–	50 NSC	–*
50 AREG⁻/⁻	–	50 NSC	–*
50 AREG⁻/⁻	–	50 NSC	++
50 AREG⁻/⁻	–	50 NSC	++
50 AREG⁻/⁻	–	50 NSC	++
50 AREG⁻/⁻	–	50 NSC	++
50 AREG⁻/⁻	–	50 NSC	–
50 AREG⁻/⁻	–	50 NSC	–
Tgen2- GFP⁺Areg⁻/⁻ Chimeras	–	–	++ (7)*; –(3)*

Results of transplantation of AREG+ and AREG⁻/⁻ mammary cells mixed with either WAP-Cre/Rosa26LacZ+ (WCR26/LacZ⁺) testicular cells or GFP-positive neural stem cells (NSC). The host strain was athymic Nu/Nu. Outgrowth: –, none; +, rudimentary; (<10% fat pad filled); ++, low (10–50% fat pad filled); +++, substantial (>50% fat pad filled). *results after a single round of pregnancy, lactation and involution.

cells were located along the ductal structures (yellow arrows). These results suggest that basally derived cells may be important in the expansion of mammary lobules during pregnancy, and that AREG⁻/⁻ cells may be unable to redirect neural stem cells to adopt a myoepithelial cell fate.

DISCUSSION

Our studies clearly support the conclusion that AREG signaling in the mammary epithelium is not required for the conversion *in vivo* of seminiferous tubule cells and neural stem cells to differentiated mammary epithelial cells. On the other hand, there was no detectable rescue of the ductal elongation defect in AREG-null mammary epithelium by the converted cells, unlike progesterone receptor null MEC-converted non-mammary cells, which supported alveolar development by progesterone receptor-knockout MECs (Bruno et al., 2014). It was shown (Ciarloni et al., 2007) that AREG⁻/⁻ mammary cells could be incorporated in transplanted mammary epithelial outgrowths when mixed with WT MECs. Therefore we speculate that AREG⁻/⁻ cells, while capable of differentiation when in contact with WT cells, are unable to redirect non-mammary cells to AREG-positive MECs capable of producing cap cells, which are required for ductal penetration of the mammary fat pad at the growing tips of TEBs. This finding indicates that separate regulator(s) are required for conversion of non-mammary cells into mammary other than the regulator(s) necessary for initial cell division during puberty and ductal elongation via TEBs. Our previous work has demonstrated that mammary cells that have knockouts in progesterone, progesterone receptor, SMA and AREG are all capable of redirecting non-mammary stem cells to a mammary cell fate; thus, individually all of these genes are dispensable for redirection. Mixing studies using non-mammary cells with ERα⁻/⁻ epithelium proved less successful, mainly because this epithelium failed to proliferate in post-pubertal WT hosts, either during pregnancy or in its absence. This finding is the first to suggest that ERα is a necessary factor for non-mammary cell reprogramming, either directly through ER signaling or indirectly as a result of ERα-dependent epithelial cell proliferation. AREG, a downstream mediator of ER signaling, is not necessary for reprogramming, but, like ERα, is necessary for ductal growth and elongation.

Immunohistochemical studies indicated that the non-mammary cells expressed mammary epithelial-specific antigens after incorporation into chimeric mammary outgrowths, including hormone receptors and proteins necessary for milk production and expulsion. As the primary function of the mammary gland is to produce milk, the finding that reprogrammed cells are capable of forming mature milk-producing structures demonstrates a complete functional conversion into a mammary cell fate. Indeed, these characteristics were conserved in the secondary outgrowths produced by implantation of fragments of the original chimeric outgrowth, demonstrating that the re-directed non-mammary cells not only retain mammary epithelial traits, they also are capable of cell division and self-renewal.

In our earlier examination of re-directed neural stem cells in chimeric mammary outgrowths, we observed some of the re-directed cells retained expression of nestin (Booth et al., 2008). However, in the present work, we did not detect nestin staining in the re-directed GFP-positive neural cells. Nevertheless, nestin was detected in the first and second generation outgrowths in pregnant hosts. The staining was present in cells at the periphery of these chimeric growths and was not colocalized with GFP expression. It has been reported that nestin staining in intact mammary epithelium is restricted to basally located cells (Li et al., 2007). Nestin staining was also observed at the periphery of alveolar
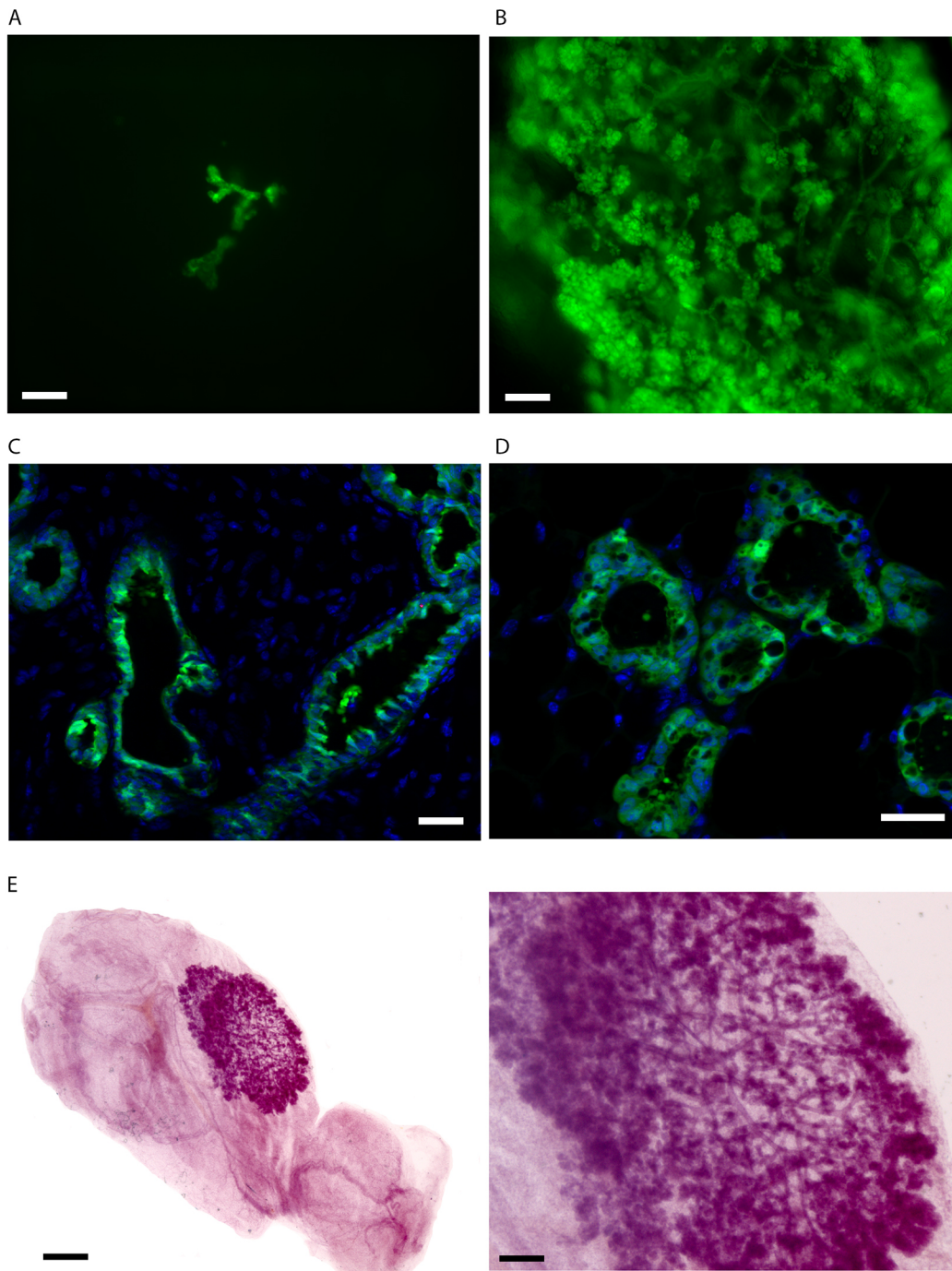
Fig. 2. AREG$^{-/-}$ mammary cells are capable of reprogramming GFP-positive neural stem cells. (A,B) Outgrowths from AREG$^{-/-}$ mammary cells transplanted with neural stem cells constitutively expressing GFP show small rudimentary GFP-positive ductal outgrowths in virgin recipient fat pads (A) and larger alveolar development upon pregnancy (B). (C,D) Cross-sections of constitutive GFP-positive chimeric outgrowths demonstrate limited contribution for GFP-negative AREG$^{-/-}$ cells to epithelial structures (C, virgin; D, parous). (E) Carmine Alum staining confirmed that the predominate epithelial growth was due to the redirected GFP-positive neural stem cells. Scale bars: 0.5 mm (A,B); 100 µm (C,D); 1 mm (E, left), 0.5 mm (E, right). Staining images are representative of two glands per group, with staining performed in triplicate; total numbers of replicates are shown in Tables 1 and 2.

hyperplastic outgrowths suggesting that basal cell-derived nestin-expressing cells may be important in the penetration of the fat pad by alveolar structures. AREG$^{-/-}$ cells may be unable to redirect neural cells to adopt a myoepithelial cell fate. This is different to what we previously found for neural cells marked conditionally with LacZ mixed with AREG-positive MECs (Booth et al., 2008), as those cells differentiated into myoepithelium during pregnancy. It is unlikely that this difference is related to the difference in

reporters. It is more probable that AREG is necessary for cap cell differentiation and the formation of the TEBs, which are required both for penetration of the fatty stroma and the formation of SMA-positive myoepithelial cells lining the subtending mammary ducts.

The significance of our results is that estrogen signaling through AREG is indispensable for post-pubertal ductal growth in the mouse but is not required either for alveolar development and function, or
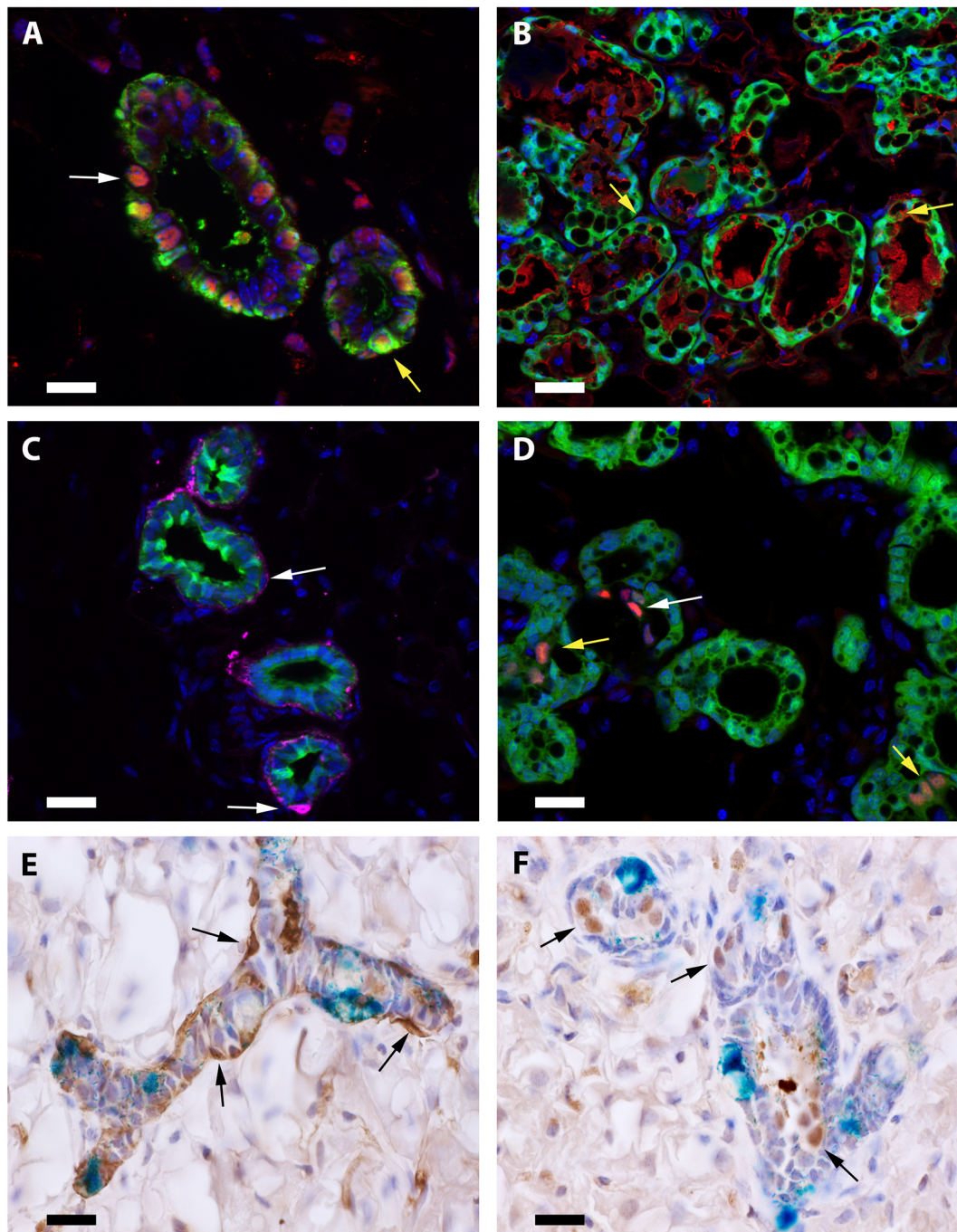
Fig. 3. Reprogrammed mammary outgrowths express typical mammary proteins. Outgrowths from AREG$^{-/-}$ mammary epithelial cells transplanted with constitutive GFP-positive neural stem cells (A–D) and Wap-Cre/Rosa26-LacZ+ male testicular cells (E,F) were stained for ERα (A), casein (B, arrows show intracellular casein staining), SMA (C,E), and progesterone receptor (D,F). White arrows denote positive staining of GFP-negative, AREG$^{-/-}$ mammary cells. Yellow arrows denote positive staining of GFP-positive reprogrammed cells. Black arrows denote positive staining of LacZ-negative mammary cells. Hormone receptor positive cells were composed of both AREG$^{-/-}$ mammary cells as well as reprogrammed cells. SMA-positive cells did not express the respective reporter genes suggesting they were not from WAP-Cre activated LacZ-positive cells or GFP-positive precursors. Scale bars: 20 μm (A,C,E,F); 40 μm (B,D). Staining images are representative of two glands per group, staining performed in triplicate, total replicates found in Tables 1 and 2.

for re-direction of non-mammary cells to mammary epithelial cell fates. It remains a puzzle why the absence of ERα signaling alone results in the absence of growth and alveolar development during pregnancy. This obviously requires estrogen signaling to other genes, whose activity is required for secretory lobular development. Among these genes are those encoding progesterone receptor, RANKL (also known as TNFSF11) and its cognate receptor, and

ELF5 (Brisken and Ataca, 2015). AREG is detected among the cells in the chimeric outgrowths, as is progesterone receptor and ERα. This suggests that ERα signaling is directed toward genes in the surrounding stroma that have not yet been identified, since the re-directed non-mammary cells and the AREG-null tissues alone form alveoli that appear capable of full functional differentiation in the absence of the AREG gene.

MATERIALS AND METHODS
Mice
Female athymic NCr Nu/Nu mice were used for transplantation studies (Charles River). AREG$^{-/-}$ and ERα$^{-/-}$ mice were as previously described (Luetteke et al., 1999; Mallepell et al., 2006). The transgenic WAP-Cre/Rosa26-LacZ+ mice were engineered and typed according to Wagner et al. (2002). All mice were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The National Cancer Institute (NCI) Animal Care and Use Committee approved of all experimental procedures.

Sperm and germ cell dissociation procedure
Testes were excised from WAP-Cre/Rosa26-LacZ+ males as previously described (Bellve et al., 1977) with a few modifications. The testes were decapsulated to remove the tunica albuginea, placed in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA) containing 0.5 mg/ml collagenase (Sigma-Aldrich, St Louis, MO), and incubated at 33°C while shaking for 15 min at 120 rpm. The seminiferous tubules were pelleted by centrifugation at 1200 rpm (∼100 g) for 5 min. The fatty top layer and the pellet were washed twice in DMEM and dispersed seminiferous tubules were then placed in 20 ml DMEM containing 0.5 μg/ml trypsin and 1 μg/ml DNase (Invitrogen) and incubated as above. Remaining cell aggregates were triturated by pipetting 10–12 times. Cells were pelleted and washed as above, then resuspended in 10 ml DMEM with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and passed through a 40 μm filter to remove remaining clumps. Viability was determined by Trypan Blue exclusion, and cell counts were determined using a hemocytometer.

Neural stem cell isolation
Isolation of neural stem cell (NSCs) was performed using an established protocol, based on selective expansion, for the isolation and expansion of NSCs from the fetal and adult mice brains. After dissection, triturated tissue was plated in culture medium containing apotransferrin (for iron transport), insulin (as a pro-survival signal) and basic fibroblast growth factor (mitogenic for NSCs) (Johe et al., 1996), which supports the expansion of stem cells but not other cell types.

Fetal cultures are normally passaged approximately every 5 days. After the first passage (which removes most of the remaining contaminants), the cultures are composed of 95% NSCs. Clonal and real-time lineage analyses confirmed their self-renewal properties and multipotential. Adult cultures are composed of both multipotent (Johe et al., 1996; Androutsellis-Theotokis et al., 2006) stem cells and a glial-restricted progenitor, but their morphologies are distinct, and because they are cultured under clonal conditions, they are easily distinguished.

Fetal cultures are derived from embryonic day (E)13.5 mouse embryo cortex, whereas adult cultures are derived from the subventricular zone of 2- to 3-month-old mice. After dissection, the culture conditions were identical for these two stem cell sources, and cells were then maintained as previously described (Booth et al., 2008).

Mammary epithelial cell dissociation
Mammary glands were harvested and dissociated with 0.1% collagenase (Sigma) overnight at 37°C in complete tissue culture medium with 10% fetal calf serum. The following day, samples were triturated with a 10 ml sterile pipette and passed through a 19 gauge needle. Resulting organoids were cultured in plastic flasks in DMEM supplemented with 10% fetal bovine serum, insulin (1.0 μg/ml) and epidermal growth factor (10 ng/ml). Fibroblasts were removed by differential trypsinization after 72 h and mammary epithelial cells collected 24 h later (Smith, 1996).

Mammary fat-pad clearing
The surgical techniques used to clear the mammary fat-pads of epithelium were performed as previously described (Deome et al., 1959; Boulanger et al., 2007). Briefly, 3-week-old female Nu/Nu mice were anesthetized, and the clearing procedure was performed immediately before the insertion of transplanted tissue fragments or injection of cell suspensions. Cell suspensions were injected in 10 μl volumes of non-supplemented DMEM with a Hamilton syringe

equipped with a 30 gauge needle. For second-generation outgrowths, small mammary tissue fragments (1–2 mm^2) from primary outgrowths were inserted into a small pocket in the fat pad created using watchmaker forceps.

X-gal staining of mammary and testicular whole mounts
The #4 inguinal fat pads of mice were excised from the transplant-bearing mice, spread on glass slides, and fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature. Glands were then permeabilized in 0.02% NP-40, 0.01% sodium deoxycholate and 0.002 M MgCl$_2$ in phosphate-buffered saline (PBS) overnight at 4°C and processed for X-gal (Wagner et al., 1997). For X-gal controls, intact host glands were treated identically. Stained glands were repeatedly rinsed in PBS and postfixed with Carnoy's fixative. Glands were then dehydrated in a graded series of alcohol and cleared in xylene before analysis.

Preparation of non-fluorescent mammary gland whole mounts
Excised entire inguinal fat pads were mounted onto glass slides and spread to expose the maximum surface area for improved viewing. Glands were then placed in Carnoy's fixative (1:3:6 ratio of acetic acid, chloroform and ethanol) for 4 h at room temperature. They were then stained with Carmine Alum, dehydrated through a series of alcohols, cleared in xylene, and sealed with Permount and a glass coverslip.

Preparation of fluorescent mammary gland whole mounts
GFP-positive whole-mounted glands were visualized as previously described (Landua et al., 2009). Briefly, glands containing mammary–NSC chimeras were excised and spread on a glass slide and fixed in 4% PFA for 2 h at 4°C. Slides were then incubated in 50% (v/v) glycerol in PBS overnight at 4°C. Glands were then dehydrated via treatment with 75% (v/v) glycerol in PBS followed by 100% glycerol for 1 h each at room temperature. Glands were then visualized using the Zeiss Axio Imager.M2.

DNA isolation and PCR detection
DNA was isolated from mammary cells and whole mounts according to the 'animal tissues spin column protocol' as part of the Qiagen DNeasy Blood and Tissues Kit (cat. no. 69506; Valencia, CA). PCR analysis for detection of the *Sry* gene was performed using the following primers: forward 5′-G-CTGGGATGCAGGTGGAAAA-3′; reverse 5′-CCCTCCGATGAGGCT-TGATATT-3′ (product=125 bp). Detection of the *Areg* gene was performed using the following primers: forward 5′-GACAATGGCCGTGACCTCTCT-3′; reverse 5′-TGTCATCCTCGCTGTGAGTC-3′ (product=175 bp). DNA was amplified under the following conditions: 95°C for 5 min; 30 cycles of: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; then 72°C for 7 min. Amplified DNA was loaded into 2% agarose gels containing 0.5 μg/ml ethidium bromide (Invitrogen 15585-011), electrophoresed at 100 V and visualized under UV light.

Immunohistochemical staining of mammary tissues
For histological examination, X-gal-positive glands were embedded in paraffin and cut into 5 μm sections and mounted on positively charged slides. Sections were subsequently cleared in xylene and rehydrated through ethanol gradients. Antigen retrieval was performed by heating slides in a boiling water bath for 20 min in either 10 mM citrate buffer pH 6.0 (Dako, Capenteria, CA) or Tris-EDTA pH 9.0 (Dako). Incubating slides in 3% hydrogen peroxide for 15 min at room temperature blocked endogenous peroxidase activity. Slides were blocked with normal horse serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature and then incubated overnight with primary antibodies at 4°C. Primary antibodies included those against progesterone receptor (1:75; A0098, Dako), smooth muscle actin (1:100; 18-0106, Zymed), and casein (1:1000) and nestin (1:50) as previously described (Smith and Vonderhaar, 1981; Booth et al., 2008). Slides were then washed three times and secondary antibody staining was performed using the RTU Vectastain (goat anti-rabbit-IgG and mouse-IgG) kit (Vector Laboratories). Staining was visualized using the DAB peroxidase substrate kit (Vector Laboratories) according to the manufacturer's recommendations. Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich) and negative tissue controls were included in all immunohistochemical analyses.

For histological examination of GFP-positive outgrowths, glands were rehydrated in gradient washes of 70%, 50% and 30% (v/v) glycerol in PBS, placed in OCT compound (Tissue-Tek), and snap-frozen for embedding. Glands were then sectioned at 10 μm and stored at −80°C. For staining, slides were allowed to warm to room temperature for 30 min and then fixed in pre-chilled (−20°C) acetone for 10 min at room temperature. Slides were then allowed to briefly air dry before being washed three times in PBS for 5 min each time. Sections were blocked in a humidified chamber for 1 h at room temperature in PBS containing 1% BSA and 10% goat serum, and then incubated overnight with primary antibodies at 4°C. Primary antibodies that were used those against progesterone receptor, smooth muscle actin, casein and nestin (all antibodies used as above) as previously described (Smith and Vonderhaar, 1981; Booth et al., 2008). Slides were then washed three times and secondary antibody staining was performed using Alexa Fluor 568, and Alexa Fluor 647-conjugated secondary antibodies (Life Technologies, Eugene, OR). Slides were mounted using Prolong Gold Antifade Reagent (P36931, Life Technologies) as per manufacturer's recommendations.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.L.G., C.B., G.S.; Methodology: A.L.G., C.A.B., L.H.A., S.C., G.S.; Validation: A.L.G.; Formal analysis: C.A.B., L.H.A., S.C.; Investigation: A.L.G.; Writing - original draft: A.L.G., C.B., G.S.; Writing - review & editing: A.L.G., C.A.B., L.H.A., S.C., C.B., G.S.; Supervision: G.S.; Project administration: C.B., G.S.

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