Published in final edited form as:

Exp Cell Res. 2010 February 1; 316(3): 422–432. doi:10.1016/j.yexcr.2009.11.006.

Amphiregulin mediates self-renewal in an immortal mammary epithelial cell line with stem cell characteristics

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Abstract

Amphiregulin (AREG), a ligand for epidermal growth factor receptor, is required for mammary gland ductal morphogenesis and mediates estrogen actions in vivo, emerging as an essential growth factor during mammary gland growth and differentiation. The COMMA-D β -geo (CD β geo) mouse mammary cell line displays characteristics of normal mammary progenitor cells including the capacities to regenerate a mammary gland when transplanted into the cleared fat pad of a juvenile mouse, nuclear label retention, and the capacity to form anchorage-independent mammospheres. We demonstrate that AREG is essential for formation of floating mammospheres by CD β geo cells and that the mitogen activated protein kinase signaling pathway is involved in AREG-mediated mammosphere formation. Addition of exogenous AREG promotes mammosphere formation in cells where AREG expression is knocked down by siRNA and mammosphere formation by AREG^{-/-} mammary epithelial cells. AREG knockdown inhibits mammosphere formation by duct-limited mammary progenitor cells but not lobule-limited mammary progenitor cells. These data demonstrate AREG mediates the function of a subset of mammary progenitor cells in vitro.

Keywords

Amphiregulin; mammary; mammosphere; progenitor cell

INTRODUCTION

Many factors are involved in the process of mammary gland ductal morphogenesis including members of the epidermal growth factor (EGF) family, the ovarian hormones estrogen (E2) and progesterone (P), Wnt-4 and insulin-like growth factor (IGF)-2 [1–3]. These factors act through paracrine mechanisms with signals originating from somatic stem cells and adipocytes

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and other cell types from the surrounding mammary fat pad, including the neural, lymphoid and endothelial cells. The proteolytic release (shedding) of the EGF family member amphiregulin (AREG) from the epithelium and subsequent paracrine activation of the EGF receptor (EGFR) in the surrounding stroma is essential for mammary development [4]. AREG is the most abundant EGF-family member during the pubertal expansion of the mammary gland [5]. AREG is induced by and required for estrogen mediated epithelial proliferation, terminal end bud formation and ductal elongation in the mammary gland [6].

A property associated with stem/progenitor cells is the capacity to form colonies when grown as free-floating sphere cultures in anchorage-independent culture conditions. The free-floating colonies that form are termed mammospheres based on the neurosphere culture system that was established previously [7]. The hypothesis behind the neurosphere and mammosphere culture systems is that stem cells are able to survive and self-renew when contact with the basement membrane and extracellular matrix is disrupted whereas differentiated cells experience anoikis and die. The immortalized cell line COMMA-D β -geo (CD β geo) was derived from its parent line COMMA-D, a cell line that was formed from mid-pregnant Balb/c murine mammary tissue [8]. This was accomplished by selection of a dominant selective gene transfer [9]. In this report we present data demonstrating that CD β geo cells function as murine mammary epithelial progenitor cells and form mammospheres in vitro. The formation and expansion of these spheres is regulated by the growth factor AREG and the mitogen activated protein kinase (MAPK) signal transduction pathway. AREG regulates the expansion of the duct-limited subtype of mouse mammary progenitor cells.

MATERIAL AND METHODS

Cell Culture

Cultures maintained in 2-dimensional culture were grown as described previously [2]. MEGM supplemented with 10% FBS, BPE, EGF, insulin, hydrocortisone and GA-1000. CD β geo cells were grown in 3-dimensional culture, as mammospheres, at a concentration of 1000 cells/ml in 6-well ultra-low attachment plates in a total volume of 3ml/well. These conditions are based on preliminary studies where 10 concentrations of cells were seeded ranging from 10 cells/ml to 250,000 cells/ml. Only at the three lowest cell densities (10, 100 and 1000 cells/ml) was evidence of cell aggregation absent. Mammospheres failed to form at 10 cells/ml. The concentration of 1000 cells/ml was chosen based on these observations. The media is comprised of 3:1 DMEM-low glucose:Ham's F-12 supplemented with EGF (20 ng/ml), bFGF (40 ng/ml), B-27 and heparin (4 μ g/ml). All cultures were maintained with 5% CO₂ at 37°C. Passaging of the mammospheres entailed collecting the media and non-adherent cells by centrifugation. The pellets were suspended in warm trypsin for 5 min followed by repeated pipetting to break up the spheres. Cells were then reseeded at 1000 cells/ml as stated above. Mammosphere numbers were collected by visual counts, any sphere consisting of at least 5 cells was counted.

Immunoprecipitation and Western analysis

Total protein was extracted via Cell Lysis Buffer (Cell Signaling Technology; Beverly, MA) supplemented with 1 mM PMSF according to manufacturer's guidelines. Protein lysates (200 μ l) or collected conditioned medium (1 ml) were incubated with primary antibodies (5 μ l, 20 μ l respectively of primary antibody at a concentration of 200 μ g/ml, anti-AREG, anti-TGF α , anti-EGFR or anti-HB-EGF) overnight at 4°C with rocking. Protein A agarose beads (20 μ l of a 50% slurry) were added and incubated for 3 hours at 4°C with rocking. The samples were washed with lysis buffer 5X then analyzed by Western blotting. Protein samples were mixed 1:1 with Laemmli Sample Buffer (Bio-Rad) and boiled for 10 min. The samples were then resolved on a 10% SDS-PAGE gel and the proteins transferred to a nitrocellulose membrane. The blot was initially blocked in 5% nonfat milk/PBS plus 0.5% Tween-20 (PBST) for 1 hour

at room temperature. The blot was then probed with selected primary antibodies depending on the antibody used for immunoprecipitation at the appropriate dilutions (1:500 or 1:1000) overnight at 4°C. The blots were washed twice with PBST and exposed to appropriate secondary antibodies (1:4000) for 45 minutes at room temperature. The blots were washed twice more with PBST and the bands were visualized using LumiGLO Reagent (Cell Signaling Technology). When required, the membranes were chemically stripped using Western-Re-Probe Reagent (Calbiochem) according to manufacturer's instructions.

X-Gal staining

Mammary glands were prepared as whole mounts in which the entire mammary inguinal gland (i.e. gland 4) was spread on a glass slide and fixed for 1–2 hrs in 4% paraformaldehyde in PBS. Tissues were washed repeatedly in PBS and processed for X-Gal as described elsewhere [10]. Cultures were fixed with 4% paraformaldehyde and processed for X-Gal followed by hematoxylin counterstain.

Nuclear labeling with 5BrdU

Freshly isolated mouse primary mammary epithelial cells or the CDβgeo cell line were seeded as outlined above. 5-bromodeoxyuridine (5BrdU) was added to the media for the first 2 days of culture allowing incorporation of the label into newly synthesized DNA after which time the media was replaced with 5BrdU-free media. Mammospheres were allowed to grow for various times after which the spheres were pelleted at slow centrifugation speed (500 rpm, Beckman tabletop centrifuge). The spheres were fixed with 4% formaldehyde and processed for 5BrdU by immunofluorescent staining with either anti-BrdU-Alexa488 or anti-BrdU-Alexa647 (Invitrogen).

Inhibitor studies

Empirical studies using a range of inhibitor concentrations were performed to determine the optimal working concentrations. The pharmacological inhibitors UO126 (specific for MEK) and AG1478 (specific for EGFR) were used at concentrations of 100 nM and 10 μM respectively. Antibodies added during culture (anti-AREG, anti-ADAM17) were added at a concentration of 0.5 μg/ml. Inhibitors or antibodies were added at initial seeding and during any media change that occurred unless otherwise noted.

AREG ELISA

AREG was quantitated from conditioned medium by commercially available AREG ELISA (R & D Systems) according to company's included protocol.

Immunofluorescent staining

All staining of mammospheres was carried out in microfuge tubes. Following fixation in 4% paraformaldehyde the spheres were washed with PBS followed by incubation with denaturing solution (0.1N HCl). After a PBS wash the samples were incubated with blocking serum. The spheres were then exposed to anti-5BrdU antibody conjugated to Alexa-647 overnight at 4°C. The spheres were washed again and resuspended in ProLong Gold antifade mounting reagent with DAPI, spotted onto slides and coverslipped. Confocal images were captured using a Zeiss NLO confocal microscope with postproduction using Adobe Photoshop.

Tissue transplantation

DeOme and colleagues first described the technique of tissue fragment transplantation into mammary fat pads cleared of endogenous mammary epithelium [11]. The surgical procedures for clearing the fat pad of 3-week-old female mice have been described previously. CDβgeo

or WAP-Cre/Rosa26 mammary epithelial cells at the desired concentration were injected (10 μ l volume) into the cleared fat pads. When cells were grown in monolayers prior to transplantation 50,000 cells were transplanted. When mammospheres were transplanted without dissociation 20 mammospheres consisting of at least 20 cells were individually selected after 7 days in anchorage-independent culture. The recipients were kept as nulliparous virgins for 7–12 weeks to allow the transplanted epithelium to penetrate the host fat pad and form a ductal tree.

All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The National Cancer Institute Animal Care and Use Committee approved all experimental procedures.

siRNA transfections

CD β geo cells were plated at a density of 2×10^5 cells/well in 6-well plates in antibiotic-free media and grown until 80% confluent. The transfections were carried out using commercially available AREG siRNA kits (Santa Cruz Biotech) according to manufacturer's guidelines. Following transfection the cells were placed in 3D culture (1000 cells/ml in 3 ml of culture media in ultra-low attachment 60mm dishes or 12 well plates) to form mammospheres as outlined above. RT-PCR using commercially available primers (Santa Cruz Biotech) was performed in order to demonstrate message knockdown.

Statistical analyses

The GraphPad Prism software package was used for all statistical analyses. Data were considered significant at $p < 0.05$. Representative data are presented as means $\pm$ SEM.

RESULTS

Murine mammary cells form mammospheres

A characteristic of adult somatic stem/progenitor cells is their ability to form anchorage-independent sphere cultures in vitro. This has been demonstrated with various cell types including neural and mammary epithelial stem cells [1,7,12]. Freshly isolated mammary epithelial cells from virgin Balb/c mice form free-floating mammospheres (5 or more cells) within 4 days (Fig 1A). The immortal CD β geo mammary epithelial cell line also has the capacity to form mammospheres in vitro (Fig 1B) indicating that this cell line, like primary tissue, possesses the progenitor cell quality of forming anchorage-independent colonies. After 4 days in culture the number of spheres that formed from both cell sources was equivalent (Fig 1C) indicating that CD β geo cells provide a comparable substitute for primary mammary epithelial cells in a mammosphere model (209 ± 11 for Balb/c, 218 ± 31 for CD β geo cells). The number of spheres remained equivalent after 7 days in culture (394 ± 8 for Balb/c, 411 ± 43 for CD β geo cells) (Fig 1C).

Somatic stem/progenitor cells facilitate postnatal development (ductal and lobular morphogenesis) of the mouse mammary gland through symmetric (proliferation) and asymmetric divisions (differentiation). Asymmetric division is characterized by the retention of the template DNA strands within the stem/progenitor cell while the daughter cell receives the newly synthesized strands of each chromosome [13]. This process can be marked with nucleic acid labels such as [3 H]-thymidine or 5-bromo-deoxyuridine (5BrdU). Mammary epithelial stem/progenitor cells utilize asymmetric division [14–16]. In the mammosphere culture system CD β geo cells exhibit this property. 5BrdU was added for the first 2 days followed by a chase period of at least 14 days after which a number of cells within the spheres retained 5BrdU (Fig. 1D). Most of the mammospheres contained one 5BrdU-retaining cell

(89%) with the maximum of three 5BrdU-retaining cells observed in any single mammosphere. This retention of nuclear label shows the incorporation of the 5BrdU into the cell's genome. After passaging of the mammospheres by dissociating of the mammospheres and reseeding them as single cells, a fraction of cells within the newly formed mammospheres contained 5BrdU. The mammospheres of the second passage were not exposed to additional 5BrdU indicating that the cells retained the nuclear label that was incorporated the label during the first 2 days of the initial seeding and proliferated after passaging. Since the second passage spheres grew from single cells this indicates that the label retaining cells divided asymmetrically and segregate new and old DNA strands. Not all of the mammosphere-initiating cells would be expected to incorporate nuclear label into their genomes as some mammospheres may have formed following the removal of the 5BrdU from the surrounding media.

CD β geo mammospheres contain mammary progenitor cells

It has been previously reported that the COMMA-D cell line has the potential to reform a mammary gland upon transplantation into the cleared mammary fat pad of a pre-pubescent mouse [9,17]. We demonstrate that the CD β geo cell line, when grown as mammospheres, maintains the ability to regenerate mammary glandular structures in vivo. CD β geo mammospheres were grown for 7 days in vitro prior to their transplantation into the cleared fat pads of 3-week old Nu/Nu female mice. After 8 weeks the reconstituted mammary glands were harvested and processed for X-gal. As shown in Fig. 2A, CD β geo cells that constitutively express *lacZ*, contribute progeny to the reconstitution of the mammary gland (the formation of a branching ductal system). At higher magnification CD β geo progeny are observed in luminal positions in the subtending ducts and developing acinar structures (Fig. 2B). Previous studies have demonstrated that the CD β geo line contains a population of basal progenitors that express p63 and keratins 5 and 14, all markers of myoepithelial cells [9]. The regenerated glands show a normal mammary epithelial architecture comprised of both luminal and myoepithelial cells. In addition, CD β geo progeny are found as ductal luminal cells and alveolar luminal cells. The luminal CD β geo progeny are able to differentiate into estrogen receptor- α (ER α) (Fig. 2C) and progesterone receptor (PR) (Fig. 2D) expressing cells. CD β geo mammospheres thus possess competency to produce normal-appearing mammary epithelial outgrowths comprised of multiple epithelial cell types. The implanted mammospheres produce large primary ducts as well as the side branches and tertiary ducts and have the capacity to develop terminal end buds, including the specialized cap cells. Even though the mammary outgrowths are formed solely by CD β geo progeny not all of the cells within the regenerated mammary gland react positively to the X-gal enzymatic assay. This is thought to be due to technical issues as it has been documented previously that the X-gal reaction is not as sensitive as antibody detection such as an immunohistochemical assay because not all of the β -galactosidase is active enzyme [18]. Mammospheres formed from mammary cells isolated from a parous WAP-Cre/Rosa-lox-STOP-lox-LacZ transgenic mouse in which the Cre recombinase has activated the Rosa26 reporter gene resulting in the constitutive activation of *lacZ* in multipotent parity identified mammary epithelial cells (PI-MEC) [19] gave similar results with the *lacZ*⁺ cells in luminal and myoepithelial positions in the mammary outgrowths (Fig 2E and F).

Mammosphere formation is MAP kinase dependent

In order to characterize further the formation of mammospheres by CD β geo cells we investigated potential signaling pathways that might be involved in the formation and maintenance of mammospheres. To determine if a specific intracellular signal transduction pathway such as the mitogen activated protein kinase (MAPK) pathways is required for mammosphere formation, a specific pharmacological inhibitor of the pathway, U0126, was added to the media throughout mammosphere formation. The MAPK pathway is known to

function in the proliferation and differentiation of many cell types. U0126 specifically binds to the MAPK pathway proteins MEK1 and MEK2 blocking downstream phosphorylation of the ensuing signaling proteins. Identical numbers of cells (1000 cells/ml) were seeded with and without the inhibitors and the number of spheres that formed was determined after 4 and 7 days in culture. Again only mammospheres consisting of 5 or more cells were considered. Figure 3A demonstrates that the numbers of mammospheres formed by cells cultured from normal Balb/c mammary tissue in the presence of the MAPK inhibitor were significantly reduced when compared to the number of mammospheres formed in media alone. Untreated mammary epithelial cells formed ~100 mammospheres after 4 days in culture and ~200 after 7 days in culture while inhibition of MAPK decreased these numbers to ~60 and 150 mammospheres ($p<0.05$). CD β geo mammosphere formation was also inhibited by U0126 after 4 and 7 days in culture (Fig 3B). Untreated CD β geo cells formed ~130 mammospheres after 4 days in culture and ~220 after 7 days in culture while inhibition of MAPK decreased these numbers to ~50 and 120 mammospheres ($p<0.05$). These results indicate that CD β geo cells behave similarly to wild type Balb/c mammary epithelial cells and that the MAPK pathway inhibitor reduces mammosphere formation.

CD β geo mammospheres release AREG

The EGF family of growth factors has long been known to play a major role in mammary epithelial growth and differentiation [20–21]. The EGF family member AREG is required for mammary ductal morphogenesis [4] and acts as a mediator of estrogen receptor- α (ER α) function [6]. AREG is initially translated as a membrane anchored protein that undergoes proteolytic cleavage resulting in the release of the mature growth factor. The cleavage is performed by the metalloproteinase ADAM17 in vitro [22–23]. We found AREG in the conditioned medium from CD β geo mammospheres grown in culture for 4 days but not in the control medium (Fig 4A). Control medium is fresh medium that has had no contact with cells. We did not find the other EGF family members TGF α (Fig. 4A) or HB-EGF (not shown) in either media. EGF is a component of the culture medium so it was present in control medium.

Role of MAP kinase in release of AREG

Next, the MAPK pathway mediated release of AREG from CD β geo mammospheres was investigated. Conditioned media of inhibitor treated and untreated CD β geo mammospheres were analyzed by ELISA. Media collected from U0126-treated mammosphere cultures contained significantly less AREG than media collected and analyzed from untreated control mammospheres (Fig 4B). Since U0126 significantly reduced mammosphere formation (Fig 3) the reduction in AREG in media from U0126 treated mammospheres is due to the decrease in mammosphere numbers. To demonstrate that the pharmacological inhibitor is acting as expected, protein analyses were conducted on the lysates collected from the treated and untreated mammospheres. A decrease in phosphorylated Erk 1/2 was found in mammospheres treated with the MEK inhibitor U0126 compared to untreated spheres (Fig. 4C).

AREG binds to the EGFR and initiates downstream signal transduction pathways [Reviewed in 24]. EGFR function was inhibited with the pharmacological inhibitor AG1478. AG1478 attenuated mammosphere formation (Fig 5A) and but also resulted in an increase in AREG in the conditioned medium after 4 days (Fig 5B). This observation indicates that signaling through EGFR is required for mammosphere formation. Unlike inhibition of the MAPK pathway where both mammosphere numbers and shed AREG were decreased inhibition of the EGFR only reduced mammosphere numbers and not the levels of AREG in the surrounding media. As the MAPK pathway is downstream of EGFR this result indicates that additional signaling pathways are involved. Fewer mammospheres formed in the presence of the anti-AREG than in untreated medium (Fig 5A) although the levels of AREG in the surrounding medium were much greater those of untreated cells after 4 days in culture (Fig 5B). These findings indicate that the anti-

AREG prevents the binding of the ligand to its receptor but not the shedding of the growth factor. While levels of AREG in treated cultures returned to control levels by Day 7 the number of mammospheres in treated cultures did not reach control levels. Indicating that AREG is essential to initiate mammosphere formation. Without sufficient AREG at the onset of mammosphere formation CD β geo cells did not form equivalent numbers of mammospheres as untreated controls even as levels of AREG in the surrounding media approached control levels in the subsequent days in culture. An antibody directed against ADAM17, the metalloproteinase responsible for the cleavage and release of AREG from its membrane-tethered state to mature growth factor, had no effect on mammosphere formation after 4 days in culture (Fig 5A). An attenuation of mammosphere formation was observed after 7 days of treatment (Fig 5A) but no significant change in AREG levels in the medium (Fig 5B). Although levels of shed AREG were initially increased following anti-ADAM17 treatment the levels dropped to control levels. After 7 days the numbers of mammospheres that formed did not change. This suggests that ADAM17 is required for the cleavage of additional factors essential for mammosphere formation in addition to AREG.

AREG enhances mammosphere formation

In order to directly implicate AREG in mammosphere formation CD β geo cells were transfected with commercially available siRNA designed to suppress AREG expression. CD β geo cells transfected with the siRNA had diminished levels of AREG protein both in the cellular lysates (Fig. 6A) and in surrounding media (Fig. 6B) when compared to control non-transfected cells that received only the transfection agent. The transfected CD β geo cells also formed fewer mammospheres (220 ± 17 for siRNA, 310 ± 40 for control; $p < 0.05$) when seeded in identical numbers as the non-transfected cells (Fig 6C). AREG mRNA levels were also suppressed in the transfected CD β geo cells compared to control CD β geo cells (data not shown).

The mammosphere forming potential of the siRNA treated CD β geo cells was restored by the addition of exogenous AREG. Figure 6D illustrates that in CD β geo cells that received siRNA mammosphere formation was inhibited but the capacity to form mammospheres was fully restored when the siRNA treated cells received exogenous AREG. In mammospheres formed from CD β geo cells that did not receive the siRNA treatment exogenous AREG treatment resulted in a statistically significant increase in mammosphere number when compared to untreated cultures. AREG not only rescues siRNA treated cultures but also induces an overall increase in mammosphere number formed by CD β geo cells.

AREG^{-/-} mammary tissue is unable to grow when implanted into wild type mammary fat pads suggesting that AREG is essential for the function of a mammary progenitor cell type [6]. Adding exogenous AREG to AREG^{-/-} mammary epithelial cells for 7 days increased the number of mammospheres that formed by 100%, (227 ± 50 compared to 100 ± 14 for untreated (Fig. 7A). Numbers of mammospheres did not increase when AREG was not added until the 4th day of culture, for 3 or 7 days (Fig. 7A and not shown), indicating that the presence of AREG in the medium at seeding is essential for mammosphere formation.

The mammospheres that formed from AREG^{-/-} cells, both treated with exogenous AREG and untreated, were dissociated into single cell suspensions and reseeded as a second passage. All cells that were recovered from the primary mammospheres were reseeded at the original density used, 1000 cells/ml. After 7 days in culture the numbers of mammospheres that formed were nearly identical to those that formed initially, 110 ± 10 untreated and 210 ± 36 for 7 day AREG treated mammospheres (Fig. 7B). These results show that AREG is required at the time of seeding for mammosphere initiation. The mammospheres formed here do not represent and expansion of stem cells but instead represent the asymmetric expansion of mammary epithelial cells. The numbers of mammospheres that formed after dissociation and passaging are

essentially equivalent to those of the first passage indicating no expansion of the stem cell population has occurred.

Mouse mammary stem/progenitor cells are classified into three types: duct-limited, lobule-limited and fully competent progenitor cells [25,26]. CD β geo cells that represent each of the limited progenitor cell subtypes have been isolated and transplanted repeatedly to form representative outgrowths consisting of mammary lobules (Figs. 8A and B) or mammary ducts (Figs. 8C and D). The initial limited outgrowths that formed were the result of transplanted cells from clones isolated from the parent CD β geo population. AREG $^{-/-}$ mice do not develop mammary ductal trees during puberty but milk-producing lobules form during pregnancy indicating a role for AREG in mammary ductal development. To address whether AREG inhibition effects duct-limited mammosphere forming progenitors isolated duct-limited and lobule-limited CD β geo cells were treated with AREG siRNA. Following AREG siRNA treatment mammosphere formation by duct-limited mammary epithelial progenitors (130 ± 21) was inhibited over two-fold compared to untreated duct-limited CD β geo cells (261 ± 41) after 4 days of culture (Figs. 8E). Levels of shed AREG in the surrounding media were also reduced in response to siRNA treatment (Fig. 8G) as well AREG mRNA levels (not shown). Lobule-limited CD β geo cells shed significantly lower amounts of AREG than duct-limited or unlimited CD β geo cells and AREG-specific siRNA resulted in undetectable levels of AREG in the surrounding media. The AREG siRNA did not have a statistically significant effect on lobule-limited progenitors (208 ± 28) nor did a scrambled control siRNA (237 ± 18). These results were equivalent after 7 days in culture (Fig. 8F). These data indicate that AREG has a role in the regulation of mammosphere formation by CD β geo duct-limited mammary progenitor cells.

DISCUSSION

CD β geo cells are able to repopulate a mouse mammary gland demonstrating that a population of CD β geo cells possesses mammary progenitor cell qualities. CD β geo cells and their progeny regenerate an entire mammary gland that includes all epithelial cell types: luminal ER α and PR positive and negative cells, as well as myoepithelial cells. As has been previously reported, AREG is an essential mediator of ER α function in mammary gland development [6]. It was demonstrated that transplanted AREG $^{-/-}$ cells could participate in ER α -mediated ductal development of the mammary gland only when wild type AREG $^{+/+}$ cells were co-transplanted. In these outgrowths AREG $^{-/-}$ cells were found in all cell types of mammary epithelial cells indicating their multipotent capacity. Results obtained from in vitro experiments where CD β geo cells treated with siRNA directed against AREG demonstrated this treatment impairs the formation of mammospheres, while these same cells can be rescued with exogenous AREG and form mammospheres. Likewise, when exogenous AREG was added to AREG $^{-/-}$ mammary epithelial cells mammosphere formation was dramatically increased.

Three progenitor cell types are present in the murine mammary gland: duct-limited progenitors, lobule-limited progenitors and fully competent progenitor cells [27]. Deletion of AREG specifically inhibits ductal elongation but not any preceding or subsequent developmental stage in the murine mammary gland [6] suggesting an effect on the ductal-limited progenitor cells of the mammary gland. Lobule-limited progenitors seem unaffected as demonstrated by the development of functional lobules during pregnancy in AREG $^{-/-}$ mice [6]. Since the presence of AREG enhances mammosphere formation this suggests that mammospheres formed by CD β geo cells originate from ductal-limited progenitors and perhaps the mammospheres that do form in AREG knockdown and AREG $^{-/-}$ cultures arise from lobule-limited or unlimited mammary progenitor cells within the CD β geo population.

AREG $^{-/-}$ mammosphere initiating cells are asymmetrically dividing as evidenced by the observation that the same number of mammospheres that originally formed from the initial

seeding remained constant after the spheres were dissociated and reseeded. In a study of the mammosphere forming efficiency (MFE, # of mammospheres/# cells seeded) of human ductal carcinoma in situ (DCIS) samples it was reported that DCIS samples had a MFE of 1.1% when in grown in similar conditions as the ones employed here [28]. Additionally upon dissociation they reported that DCIS-derived cells regenerated mammospheres at 85%. After dissociation AREG^{-/-} murine mammary epithelial cells that were supplemented with exogenous AREG regenerated mammospheres at 92.5%. These results indicate that mammospheres formed from initial seeding of 1000 cells/ml are clonal and not an expansion of stem/progenitor cells. Furthermore both our current study and the study examining DCIS MFE found that signaling through EGFR is essential for mammosphere formation.

Additional evidence of a progenitor cell involvement in AREG mammosphere formation is the presence of DNA label-retaining cells within the sphere populations and their continued presence in secondary mammospheres. The segregation of template DNA strands through asymmetric division occurs in stem/progenitor cells of the mammary gland [14–16]. One possible mechanism pertaining to the DNA label-retaining cells is that following dissociation and reseeded the nuclear label-retaining cell undergoes an asymmetric division or divisions and that the ensuing daughter cells proliferate and act as transit amplifying cells to expand the mammosphere's cell number.

Deletion of ADAM17, the protease that is responsible for the cleavage of AREG, blocks ductal outgrowth in vivo similarly to AREG deletion [4]. This block of AREG cleavage prevents AREG from reaching and interacting with the stroma and initiating cellular responses. In our in vitro system there is no stromal component and inhibition of ADAM17 does not inhibit mammosphere formation initially. This could be due to autocrine actions of AREG, as members of the EGF family are known to function by this manner, as well as paracrine and juxtacrine signaling [29]. Posttranslational modifications resulting in several different cell surface and soluble isoforms of AREG and ADAM17 may play a role in managing these forms. The lack of expansion of mammosphere numbers after 7 days of anti-ADAM17 compared to 4-days of treatment could be the result of an alteration in the form of AREG being shed by the CDβgeo cells or of another ADAM17 target protein.

Another potential factor that may be involved in AREG-mediated mammosphere formation and ductal development in vivo is AREG's ability to bind heparin [30–31]. Heparin is a key component in the mammosphere culture system. AREG's heparin binding distinguishes it from other members of the EGF family, with the exception of HB-EGF, and could give AREG unique signaling functions. This could explain the differences in responses to EGF and TGFα, two additional EGF family members that bind to the same receptor (EGFR) as AREG.

The only known receptor for AREG is the EGFR, which is expressed on mammary epithelial cells and mammary stromal cells. EGFR is required for normal ductal development. EGFR KO epithelial cells fail to form ducts except when transplanted into wildtype stroma [32]. Blocking EGFR and downstream signaling molecules of the MAPK pathway inhibits mammosphere formation. The EGFR-MAPK pathway is a well-documented viaduct of proliferation- and differentiation-associated intracellular signaling and is also involved in transformation and tumorigenesis in a number of tissues and cell types [33]. AREG has a potent mitogenic effect that is MAPK-dependent in numerous cell types [34]. AREG is expressed and functions in other ectodermal tissues, some that display ductal outgrowths including the lungs, and may help regulate lineage restricted progenitor cells of those tissues as well [24,35].

CONCLUSION

These results not only confirm previous finding that AREG is necessary for ductal development in the murine mammary gland but also demonstrate that mammary progenitor cells respond to AREG signals in these models. Specifically mammary duct-limited progenitor cells require AREG in order to function normally.

Acknowledgments

The authors thank Daniel Medina for the gift of the ductal-limited and lobule-limited cell lines. This work was supported by the intramural research program of the Center for Cancer Research, NCI, NIH and the state of South Carolina. LJR is supported by a Basque Government Postdoctoral Fellowship.

Abbreviations

5BrdU	5-bromodeoxyuridine
AREG	Amphiregulin
CD β geo	COMMA-D β -geo cell line
EGF	epidermal growth factor
Erk	extracellular regulated kinase
MAPK	mitogen activated protein kinase
TGF- α	transforming growth factor- α

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Figure 1.

Mammospheres form from wild type mammary glands and CDβgeo cells. A) Mammosphere that formed from cells dissociated from a virgin wild type Balb/c mammary gland after 4 days in culture. B) Mammosphere that formed from CDβgeo cells after 4 days in culture. C) Graph depicting the number of spheres formed from seeding 1000 cells/ml after 4 and 7 days in culture from wild type Balb/c mammary glands (red bars) or CDβgeo cells (black bars). D) Mammosphere containing a nuclear label (5BrdU, pink, arrow) retaining cell following a 12 day chase period indicating asymmetric division within the sphere. E) Second passage of 5BrdU-retaining mammospheres from (D) demonstrating that mammospheres contain cells that have retained nuclear label (5BrdU, green, arrow) even after dissociation and reseed

suggesting best explained by the presence of label retaining cells that have divided asymmetrically and retained originally labeled DNA strands. Nuclei stained with DAPI in D and E. Scale bars = 50µm in A and B, 20 µm in D and E.

Figure 2.

CD β geo mammospheres reconstitute mammary glands. Mammospheres that formed from CD β geo cells after 14 days in culture were transplanted into the cleared mammary fat pads of 3-week old female Nu/Nu mice. The resulting outgrowths were removed 8 weeks later, fixed, processed for Xgal staining, sectioned and counterstained with hematoxylin. A) Low power image of a mammary outgrowth demonstrating that Xgal positive (blue) CD β geo cells have contributed progeny to the reconstitution of the gland. B) Higher power image of (A) showing CD β geo cells in luminal positions in a subtending duct and developing acinar structure. C) Cross section of (A) demonstrating CD β geo cell progeny (stained for β -gal, green) differentiate into ER α expressing cells (red). D) Cross section of (A) demonstrating CD β geo cell progeny

(stained for β -gal, green) differentiate into PR expressing cells (red). Mammospheres formed from a parous WAP-Cre/Rosa26R transgenic mouse in which the Cre recombinase has activated the Rosa26 reporter gene gave similar results. The lacZ⁺ cells are located in both E) ductal and acinar luminal positions and in F) myoepithelial positions (arrows). Scale bars = 1 mm in A, 20 μ m in B, 10 μ m in C and D, and 40 μ m in E and F.

Figure 3.

Mammosphere formation requires MAPK. A) Freshly isolated Balb/c mammary epithelial cells were seeded in mammosphere forming conditions with or without UO126. The numbers of mammospheres were counted visually at 4 days and 7 days after seeding. B) CDβgeo cells were seeded in mammosphere forming conditions with or without UO126. The numbers of mammospheres were counted visually at 4 days and 7 days after seeding. *p < 0.05 vs. untreated. Error bars indicate S.E.

Figure 4.

CD β geo mammospheres release of amphiregulin is MAPK-dependent. A) Mammospheres formed from CD β geo cells were grown for 7 days at which time the media was collected and immunoprecipitation performed on control media (CtlM) and conditioned media (ConM) using anti-AREG, anti-TGF α or anti-EGF antibodies. The immunoprecipitates were analyzed by Western blotting. B) CD β geo mammospheres formed under normal conditions (CON) or in the presence of UO126 for 7 days at which time the conditioned media analyzed by ELISA for AREG (n=6, *p<0.05). C) Cell lysates from mammospheres were examined for p-Erk1/2 following treatment with the UO126 inhibitor or untreated (CON) after 7 days in culture.

Figure 5.

CD β geo mammospheres formed in the presence of media alone (CON), anti-AREG (aAREG), AG1478 (AG) or anti-ADAM17 (aADAM). A) The number of mammospheres that formed was counted after 4 and 7 days in culture. B) After 4 and 7 days the conditioned media was collected and AREG was analyzed by ELISA (n=6, *p<0.05).

Figure 6.

Transfection with siRNA directed towards AREG reduces AREG production and mammosphere formation. A) Western analysis following immunoprecipitation using anti-AREG of lysates from mammospheres that formed following AREG siRNA transfection (siRNA) or mock transfection (CON). B) Results of AREG ELISA demonstrating that siRNA decreases the levels of AREG in conditioned media. C) The number of mammospheres that form from CDβgeo cells after transfection is significantly reduced compared to control. D) The addition of exogenous AREG restores the CDβgeo mammosphere forming quality following AREG siRNA transfection. (* $p < 0.05$, scale bars = S.E.).

Figure 7.

Addition of exogenous AREG rescues mammosphere capacity of AREG^{-/-} mammary epithelial cells. A) AREG^{-/-} mammary epithelial cells were seeded in mammosphere forming conditions (1000 cells/ml) with (right bar) or without (left bar) the addition of exogenous AREG. The middle bar demonstrates that when AREG is added after the fourth day in culture there is no change in mammosphere numbers when compared to the untreated (left bar) indicating that AREG is essential for mammosphere formation at the onset of formation. B) The mammospheres that formed in (A) were dissociated and reseeded as outlined in (A). After 7 days the number of mammospheres were counted. Left bar=untreated, middle bar

mammospheres that received AREG at day 4 of culture, Right bar= mammospheres that received AREG from initial seeding. N=3, *p<0.01.

Figure 8.

A subset of mammary progenitor cells responds to AREG inhibition. Examples of mammary outgrowths following transplantation with A) lobule-limited progenitor cells and C) duct-limited progenitor cells. Panels (B) and (D) are higher magnifications of (A) and (C) respectively. Transfection with siRNA directed towards AREG reduces mammosphere formation by duct-limited progenitor cells. Duct-limited CD β geo cells (DUC-L), and CD β geo lobule-limited cells (LOB-L) were untreated (CON), transfected with AREG siRNA (siRNA) or a scrambled control siRNA (SCR) and placed in mammosphere forming conditions. The numbers of mammospheres that formed were counted after E) 4 days and F) 7 days. N=6, *p<0.01 vs. CON.