Induction of Ductal Morphogenesis and Lobular Hyperplasia by Amphiregulin in the Mouse Mammary Gland

Nicholas J. Kenney, Gilbert H. Smith, Karen Rosenberg, Mary Lou Cutler, and Robert B. Dickson

Lombardi Cancer Center, Georgetown University, Washington, DC 20007 [N. J. K., K. R., R. B. D.], and Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892 [G. H. S., M. L. C.]

Abstract
As the juvenile mouse mammary gland matures, it undergoes extensive epithelial proliferation, leading to a network of ductal branching that transverses the organ. Recent evidence suggests that the epidermal growth factor-related peptide amphiregulin (AR) may play multiple roles in the proliferation, differentiation, and neoplastic conversion of the mouse mammary gland. Using a dual approach of recombinant AR in slow-release pellets and retroviral expression of AR, we explored the roles of this growth factor in the mouse mammary gland in vivo. We first noted that recombinant AR can reestablish longitudinal ductal proliferation in growth quiescent mammary glands of ovariectomized mice. Furthermore, retrovirally transduced mammary transplants overexpressing AR developed into hyperplastic tertiary ducts and hyperplastic lobules with increased lateral branching, apparent 9 weeks after transplantation into cleared mammary fat pads. This is the first study to demonstrate that AR can reestablish the early developmental activity of ductal mammary epithelium and induce hyperplasia in vivo. These data, coupled with previous findings that demonstrated nearly universal overexpression of AR in human breast cancer and rodent mammary tumorigenesis, suggest that AR may be an important intermediary in glandular maturation and early malignant progression.

Introduction
The mouse mammary gland undergoes morphogenesis and differentiation during postnatal development (1, 2). At 4 weeks of age, ovarian hormone levels increase, which in turn signal club-shaped epithelial structures (terminal end buds) to appear and grow distally from the nipple region to fill the fat pad. During this rapid phase of growth, an extensive network of epithelial ductal branching is developed within the parenchyma (1). Another round of epithelial expansion and maturation begins slightly before implantation of the fertilized egg and ends just before parturition in the pregnant mouse. Rapid proliferation and differentiation of milk-synthesizing secretory epithelium are evident during this period (2).

Serum- and tissue-derived growth factors are thought to play an inductive role in the modeling of the mammary gland (2). Members of the TGFβ family, fatty acid-binding proteins, mammary-derived growth inhibitor, insulin-like growth factors, fibroblast growth factors, and EGF-like family members TGFα, cripto-1, and AR may act as local mitogens or inhibitors in human and mouse mammary epithelial cells. Developmental studies suggest that the expression of these genes is modulated during various stages of growth and maturation of the mouse mammary gland (2–9).

We have concentrated our efforts on AR expression and its biological role in the mammary gland. Human AR is a heparin-binding 78–84 amino acid growth factor that was initially isolated from the human breast cancer cell line MCF-7 (10). Structurally, AR has a six-cysteine motif, similarly found in most members of the EGF-like family (11). AR binds, activates, and autophosphorylates the EGFR and transphosphorylates p185erbB2 through an EGFR-dependent mechanism (10, 12).

Biologically active AR can act as a potent mitogen for nontransformed immortalized human mammary epithelial cells (11, 13, 14). A reduction in AR mRNA levels by antisense oligonucleotides reduces the proliferative rate of the immortalized, nontransformed, human mammary epithelial cell line 184 A1N4 (13). Moreover, the biological activity and receptor signaling of AR can also be modulated in mammary epithelial cells by its interaction with heparin and heparin sulfate glycosaminoglycans in vitro (14).

In vivo, AR protein is expressed during all stages of the developing mouse mammary gland. AR protein is expressed by the cap cells of the terminal end bud, myoepithelial cells, and luminal epithelial cells, and it is secreted into the mammary ductal lumen of 4–13-week-old C57 Bl and FVB mice (7). The levels of AR protein are also elevated in the mammary gland following pregnancy and lactation. Mechanistically, AR is a protein kinase C-, progesterone-, and estrogen-inducible protein, and its expression is modulated in several estrogen-responsive and

3 The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; AR, amphiregulin; O VX, ovariectomized; BrdUrd, 5'-bromo-2'-deoxyuridine; TEB, terminal end bud; MMTV, mouse mammary tumor virus; S-GAG, sulfated glycosaminoglycan; HS-GAG, heparan sulfate-GAG.

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2 To whom requests for reprints should be addressed, at Lombardi Cancer Center, Georgetown University, Washington, DC 20007. Fax: (202) 887-7505.

estrogen nonresponsive human breast cancer cell lines (15). TGF-α, neu, int-3, polyoma middle T antigen, and SV40 large T antigen transgenic mice that spontaneously produce mammary epithelial tumors all preferentially process several unique isoforms of AR protein. In addition, point-mutated c-Ha-ras or c-erbB2 transformed mammary epithelial cells, and approximately 80% of human primary breast carcinomas overexpress AR protein and mRNA (16–20).

Because previous reports indicate that dysregulated expression of AR may be a component of mammary tumorigenesis in vitro, we examined the short-term and long-term consequences of AR protein and gene overexpression. In this report, we have introduced a combination biogenetic approach (retrovirus-slow release pellet) to efficiently explore the early developmental morphological aberrations within a genetically manipulated mouse mammary epithelial population.

Results
Exogenous AR and Ductal Morphogenesis. Previous reports have demonstrated Elvax pellets that contain either recombinant EGF or TGF-α are able to stimulate ductal morphogenesis in the mammary glands of OVX mice. To compare the morphogenetic effects of AR Elvax pellets to EGF or TGF-α pellets, we repeated this approach in both C57 Bl6 and FVB mouse strains.

Our results indicate that in C57 Bl mice, AR reestablished the early developmental appearance that constituted the formation of terminal end buds and ductal morphogenesis, all of which occurred in close proximity of the pellet (Fig. 1, E and F). We also observed that in the AR group, the formation and migration of the secondary ducts closely resembled EGF implants, which served as our positive controls [Fig. 1, C (EGF) and E (AR)]. Furthermore, to determine if this effect could be reproduced in another inbred mouse strain, this method was carried out in FVB OVX female mice. We con-
Fig. 2. Percentages of BrdUrd incorporation and immunohistochemical localization of anti-BrdUrd in EGF and AR pellet-implanted mammary glands. The percentages of labeled cells for BrdUrd incorporation were derived according to "Materials and Methods." A, a histogram of anti-BrdUrd-labeled cells in the mammary ductal and end bud compartments. ■, BSA treatment; ●, EGF treatment; □, AR treatment. Bars, SE. B and C, 5-μm cross-sections of TEBs that have reappeared after EGF and AR treatment. Arrowheads, cap cells positive for anti-BrdUrd localization; large arrows, luminal epithelial cells positive for anti-BrdUrd localization. B and C, ×1000.

clude that the appearance of terminal end buds was fewer in approximate number as well as the relative size of these structures in the C57 BI6 EGF/AR groups. These results suggest that AR bioactivity in the OVX mammary gland varies with mouse strains (data not shown).

Our next approach was to analyze the amount of DNA synthesis that occurred after implantation of the AR pellet. In the sectioned glands from the C57 BI6 AR implant groups, an increase in DNA synthesis was detected in the epithelium of the end bud (22.8%) and epithelial cells that comprise the mature duct (24.2%) compared to the control BSA-treated group (2%; Fig. 2A). The percentage of DNA synthesis detected in our study was comparable to mammary glands treated with EGF, as reported previously (Ref. 3; Fig. 2, B and C). In addition, a small percentage of stromal cells (fibroblasts and adipocytes) were also positive for DNA synthesis (data not shown). Our data indicate that in addition to EGF and TGF-α, AR can also stimulate the reappearance, migration, and DNA synthesis of mammary epithelium in an ovarian hormone-free environment.

Effects of AR Expression in the Virgin Mammary Gland.

Our group has recently identified the cellular lineages that express AR in the mouse mammary gland. In both ductal and lobular compartments, AR is expressed by the myoepithelial cells, cap cells of the terminal end bud, and luminal epithelial cells in 4-week-old to 12-week-old mammary glands (7). This diverse expression pattern led us to postulate that transducing mammary epithelial cells with an AR expression vector would perturb development and significantly alter the architecture of the mammary gland. In photos from Fig. 3, C-E, we demonstrate that deregulated expression of the AR gene in mouse mammary epithelial cells results in an atypical appearance of repopulated mammary fat pads (mammary outgrowths) 12 weeks following transplantation. However, AR-transduced epithelium did not completely fill the entire fat pad area. We presume that in the migrating ductal network, AR overexpression was responsible for this effect because 95% of all AR outgrowths resulted in this appearance.

In 12 of 18 outgrowths that were visually hyperplastic, the outgrowths appeared to be quite similar to those observed
previously in the TGF-α transgenic mammary gland (21, 22). In addition, when several outgrowths were excised, sectioned, and visualized under high power magnification for pathological aberrations, we observed limited lobular hyperplasia (which is composed of greatly enlarged and dilated ductules or acini that contain proliferations of the cribriform type in Fig. 4, C and D), intraductal hyperplasia (represented as luminal epithelial proliferations within the duct), and ductal hyperplasia (indicated by hyperproliferative ducts in Fig. 3C). In addition, several fields as represented in Fig. 4C contained benign hyperplasia (enlarged ductules with columnar cells) and mild atyia not shown in these photos. We also observed a mammary tumor (intraductal papilloma) in 1 of the 12 nulliparous mice that arose 7 months following transplantation (data not shown). In contrast, no tumors arose in mice following a single round of mating. Mammary gland aberrations found in AR-transduced outgrowths are summarized in Table 2.

Upon further examination of AR mice that had hyperplastic outgrowths, an extensive array of hyperplastic lobular buds and tertiary ducts were easily identified under low magnification (Fig. 3C). We then compared these hyperplastic structures to their normal counterparts. Briefly, photos were taken from a total of 20 random fields of five AR hyperplastic outgrowths and five control pNO4 outgrowths, exposed on 5 × 7 prints, and measured for ductal distance, width, and the number of secretory lobules. Our results suggest that at high magnification, tertiary ducts from AR outgrowths were slightly more narrow (20 ± 5 μm) compared to control tertiary ducts (30 ± 5 μm; Fig. 3, B and D). Furthermore, AR outgrowths (Fig. 3D) contained a significantly high number of hyperplastic secretory lobules (20 ± 12) and secondary ducts (>9) not visually found in control pNO4 outgrowths (Fig. 3A). A comparative quantitative analysis of the width, length, distance, and the number of ducts and secretory lobules from control outgrowths and AR-transduced outgrowths are summarized in Table 1. In sum, these data demonstrate that overexpression of the AR gene can induce an architectural switch within the ductal network and pro-

Fig. 3. Whole-mount analysis of intact control pNO4- and AR-transduced mammary outgrowths 12 weeks after transplantation into the no. 4 mammary gland of 3-week-old intact mice. Mammary glands were excised, fixed, and stained according to “Materials and Methods.” A and B, pNO4-transduced outgrowths that show normal terminal ducts (Nd) with no apparent mammary aberrations. C and E, AR-transduced outgrowths that show persistent TEBs (C) and at least three types of limited hyperplasia (H) (benign, papillary, and hyperplastic lobules). D, high magnification of an enlarged area in C demonstrating focal areas of lobular hyperplasia. Bars: A and C, 200 μm; B, D, and E, 25 μm.
mot both ductal and lobular hyperplasia in the mouse mammary gland.

**AR Serial Transplants into Normal and OVX Cleared Fat Pads.** Previous studies have shown that nontransgenic and transgenic, serially transplanted mammary gland outgrowths can regenerate focal growth and undergo differentiation quite similar to their normal counterparts (22, 23). In this context, we investigated whether AR-transduced mammary tissue has the capacity to develop, migrate, and form a similar atypical appearance in normal hormonally intact or OVX mice. Therefore, we serially transplanted portions \((2 \times 10^5)\) cells of 8–14-week-old AR-transduced outgrowths into 3-week-old hormonally intact female and 5-week-old OVX syngeneic hosts.

Our observations, as illustrated in Figs. 5 and 6, and summarized in Table 2, suggest that of the 10 mice that received serial transplants, 9 contained gross pathological abnormalities comparable to those observed in the donors. However, our most interesting observation occurred in the OVX mammary gland transplants. Nine months post-transplantation, we still observed focal hyperplastic mammary lesions, persistent TEBs and regional loss of ductal spacing (Fig. 6, D and E). These results indicate that even in ovarian-compromised mammary tissue, AR overexpression can facilitate the propagation of hyperplastic mam-

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**Table 1** Morphometric characteristics of AR outgrowths\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Primary outgrowth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of branches from secondary duct</td>
<td>&gt;4</td>
<td>&gt;9</td>
</tr>
<tr>
<td>Distance between ducts (secondary to tertiary)(^b)</td>
<td>850 ± 300</td>
<td>250 ± 100</td>
</tr>
<tr>
<td>Length of tertiary duct (secondary to tertiary)(^c)</td>
<td>240 ± 100</td>
<td>130 ± 35</td>
</tr>
<tr>
<td>Width of tertiary duct (secondary to tertiary)(^d)</td>
<td>30 ± 5</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Number of hyperplastic lobules per tertiary duct</td>
<td>0</td>
<td>20 ± 12</td>
</tr>
</tbody>
</table>

\(^a\) The table is a representation of three repeat experiments of primary cultures that were transduced then transplanted according to "Materials and Methods." The table reports average duct length ± 5D. Measurements represent 10 random fields of 20 mammary ducts (either secondary to tertiary) from 5 mammary glands that were examined by whole-mount analysis, sectioned, and stained with H&E.

\(^b\) Measurement in micrometers.
As Fig. 5. Whole-mount analysis of 12-week-old control pNO4- and AR-transduced outgrowths serially transplanted into hormonally intact 3-week-old cleared mammary fat pads. Pooled portions from intact pNO4 and AR transgenic donors were transplanted according to “Materials and Methods.” In each host, the outgrowth take was 100%. The growth of control pNO4-transplanted outgrowths occupied 70% of the total fat pad area, whereas AR-transplanted outgrowths filled 40% less than its control counterpart. A and B, normal transplanted outgrowth that show normal terminal ducts (ND) and no apparent mammary aberrations. C and D, AR-transplanted outgrowth that show focal zones of hyperplasia (H). A and C, ×15; B and D, ×80.

...mary epithelium and induce deregulated ductal growth patterns.

**AR Outgrowths and the Regressed Mammary Gland.**
As mentioned earlier, serially transplanted mammary outgrowths can readily undergo functional differentiation within a syngeneic host. Recent evidence suggests that during regression of lactating transgenic TGF-α mammary epithelium, overexpression of the TGF-α gene can enhance the survival of secretory epithelium participating in apoptosis as well as contribute to the reorganization of the gland during involution (22). In this regard, we examined whether the effects of AR overexpression in the mammary gland could affect the architectural design of the gland during its natural course of reorganization following parturition. Briefly, AR fragments were transplanted into the fourth mammary glands of four 3-month-old nulliparous syngeneic female hosts with parallel cycling periods and allowed to repopulate the gland for 12 weeks. All mice were allowed to suckle their pups during these time periods. We then examined these outgrowths by microscopy to determine if there were any gross effects. Our results suggest that by whole-mount and cross-section analysis, the AR transgenic regressed gland had no apparent residual histological changes in epithelial cell death or gland reorganization. In addition, we did not detect the appearance of hyperplastic alveolar structures in the AR regressed gland, an observation which is not uncommon in the normal gland (data not shown; Ref. 24).

**Detection of the AR Protein in Intact Outgrowths.** Our laboratory reported previously that preferential processing and variable localization patterns of AR protein occur within the mammary tumors of transgenic mice overexpressing TGF-α, int-3, neu, SV40 large T antigen, and polyoma virus middle T antigen (20). To determine whether this phenomenon appeared within the AR transgenic mammary epithelium, we analyzed the distribution and expression intensity of AR protein isoforms in control and transduced mammary gland extracts by Western blot analysis, as described previously (20).
Our negative controls during these experiments were mammary gland extracts from control nontransduced outgrowths, pNO4 transduced outgrowths (empty viral vector outgrowths), and empty fat pads (epithelial-cleared fat pads). For positive controls, extracts from MMTV/int-3 transgenic mammary tumors were used. These mammary tumors are poorly differentiated adenocarcinomas that spontaneously develop as early as 7 weeks of age and express various isoforms of the AR protein (M, 32,000 and M, 21,000; Ref. 20).

Our results when analyzed by densitometric analysis indicate that relative to the negative controls, a 6.5-fold induction of the M, 28,000 and M, 8,000 AR protein occurred in the AR-transduced outgrowths (Fig. 7, arrow). In addition, when we compared the AR isoforms of control, pNO4 outgrowths to AR outgrowths, we found the same patterns of expression but not in the MMTV/int-3 tumors. We interpret these results as an indication that the preferential processing of the AR protein in the AR transgenic mammary gland in relation to previously described oncogene-induced mammary tumor tissue (20) must be influenced by the stage of malignancy (hyperplasia versus adenocarcinoma) as well as the type of germ-line mutation (i.e., int-3).

Finally, as an additional means of confirming if these outgrowths indeed contained our virally inserted expression vector, we performed Southern blot analysis on HindIII/Xhol digested genomic DNA using a 2.7-kb HindIII/Xhol fragment containing the neomycin-cytomegalovirus sequence. In control pNO4 outgrowths and AR-transduced outgrowths, but not in control nontransduced outgrowths, a 2.7-kb hybridized fragment was detected (data not shown).

Discussion
To date, the limited available data are not able to specifically define the in vivo biological pathways by which the EGF family of growth factors mediate the neoplastic conversion of the mammary gland (21, 22, 24–26). Several EGF-related peptides have been shown to play important roles in malignant progression, cell survival, metastatic potential, and
clonal expansion of the mammary gland (25). Recently, transgenic and gene targeting technology has shed light on this subject (21–22, 24). The ex vivo approach has been previously used to analyze the efficiency of retroviral transfer (β-galactosidase) or the effects of cancer-related genes such as wnt-1, c-Ha-ras, or neu (27–30). These reports demonstrate that when transplanted into cleared fatty stroma of a syngeneic host, cultured mammary epithelium can undergo morphogenesis and differentiation and can also express early and later stages of malignant progression.

In the current report, we have utilized two techniques, Elvax slow release pellets and retroviral transfer, to manipulate primary mouse mammary epithelium and to study the short- and long-term behavior of the epithelium in virgin, inviolated, and OVX female mice. We demonstrate that the short-term introduction of recombinant AR initiates ductal morphogenesis in the absence of ovarian hormones and further note that the effects of long-term overexpression of the AR gene within the mammary tree induces an atypical appearance in outgrowths within at least 9 weeks of introduction. These findings are summarized in Fig. 8.

Transduced AR primary cultures that were transplanted into a total of 30 C57 Bl6 mice resulted in 12 of 18 takes that had hyperplastic-ductallobular structures with an increase in the tertiary ducts, which were not present in control or pNO4 transplanted cultures. When the morphometric characteristics of five AR outgrowths were examined, mammary ducts (secondary through tertiary) were significantly different than control outgrowths (Table 2). This suggests that as a localized factor, AR bioactivity, can modulate the pattern of mammary ductal growth.

The Complexity of Serial Outgrowth. Using the serial transplantation approach, we have also observed that AR outgrowths present the same morphological hyperplastic appearance in mammary fat pads. However, since these outgrowths were possibly clonal in origin, the innate ability of the cells to repopulate the transplant site was only sufficient to occupy 40% of the total volume of the fat pad. We do not think that the reduction in growth of the serially transplanted AR outgrowths was due to technical difficulties (i.e., location, excision, and transplantation of an AR outgrowth) because the number of surviving transplants was 100%. On the contrary, the practice of propagating serially normal, preneoplastic, and neoplastic outgrowths has been successfully carried out for several decades. The salient observations by DeOme and later by Daniel demonstrated that through each successive transplantation, the regenerative capacity of the mammary outgrowth was reduced by about 20%, and the age of the outgrowth as well as the recipient’s mesenchyme played important roles in the formation of the mammary tree [reviewed by Kenney et al. (23)]. Whereas in our study, AR serially transplanted outgrowths invaded as much surface mesenchymal area as control outgrowths; the phenotypic changes that accrued may have been attributed to the following factors: (a) not all atypical lesions in AR-transduced outgrowths contain immortalized progenitors; and (b) the regenerative capacity of AR-transduced secretory progenitors are greatly reduced following cell renewal. The latter argument can be supported by the observations that in the regressed AR transgenic gland, we could not detect the presence of immortalized hyperplastic alveolar nodules, and in the OVX AR transgenic gland, we identified only focal

### Table 2: Pathological abnormalities observed in AR outgrowths

<table>
<thead>
<tr>
<th>Outgrowth</th>
<th>No. of transplants/take</th>
<th>Time course</th>
<th>Gross abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary transplants</td>
<td>12/18</td>
<td>8–36 weeks</td>
<td>Lobular hyperplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ductal hyperplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intraductal papilloma</td>
</tr>
<tr>
<td>Serial transplants</td>
<td></td>
<td></td>
<td>Lobular hyperplasia</td>
</tr>
<tr>
<td>intact</td>
<td>5/5</td>
<td>8–12 weeks</td>
<td>Ductal hyperplasia</td>
</tr>
<tr>
<td>OVX</td>
<td>4/5</td>
<td>4–12 weeks</td>
<td>Lobular hyperplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ductal hyperplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Persistent TEBs</td>
</tr>
<tr>
<td>Mated mice</td>
<td>4/4</td>
<td>5 and 28 days</td>
<td>None</td>
</tr>
</tbody>
</table>

*The table represents a combination of three repeat experiments from primary cultures that were transduced then transplanted according to “Materials and Methods.” Outgrowths were examined by whole-mount analysis, sectioned, and stained with H&E.

*Animal death that contained a positive take.

*Serial transplant that contained persistent TEBs and loss of ductal spacing 9 months after transplantation.

*Represents time points that mice were sacrificed during or after nursing.

![Fig. 7](image-url) Western blot detection of AR protein in control outgrowths, pNO4 (empty vector) outgrowths, AR-transduced outgrowths, empty fat pads (epithelial-cleared fat pads), and MMTV/int-3 transgenic mammary tumors. Total protein extracts were derived according to “Materials and Methods.” All lanes were loaded with 50 μg of total protein. The extracts from MMTV/int-3 tumors were used to detect various AR isoforms observed previously (20). No apparent preferential processing occurred in AR-transduced outgrowths but instead showed an 6.5-fold increase in the M, 26,000 and M, 8,000 species. Arrow, M, 26,000 and M, 8,000 species. M, molecular weight markers in thousands.
areas of hyperplastic growth compared to intact AR transgenic glands. We thus believe that because less than 10% of the committed secretory progenitors may have been transduced with AR virus, the process of immortalization to malignancy and cell renewal are distinct and separate entities. The best example thus far is that of the hyperplastic alveolar nodules, which are derived from stable premalignant lesions. Medina et al. (31) have identified an independent hyperplastic alveolar outgrowth line that reproducibly develops hyperplastic lobular lesions when propagated by serial transplant. However, of the 12 additional independent hyperplastic outgrowth lines isolated, two immortalized outgrowth lines presented primarily ductal growth that also contained TEBs and limited lateral branching. In this regard, we also observed one potent progeny that maintained its capacity to develop persistent TEBs and dysregulated ductal growth (Fig. 6, curved and open arrows). The circumstance by which the TEBs persisted in the OVX mammary gland well after 9 months posttransplantation remains quite complex.

**AR Effects on the Multipotent Stem Cell.** By definition, the TEB is a bulbous-like epithelial structure that appears at puberty and is associated with the morphogenic movement of the rudimental mammary duct during the first 2 months of development. During this period, the terminal end bud primarily consists of two types of stem cell progenitors, both of which are derived from the multipotent cap cell (1, 32): the ductal progenitor, which has the capacity to form the dense branching network of epithelial ducts that traverses the fat pad and the secretory progenitor, that has the capacity to synthesize and form milk secretory structures. Together, these cells participate in the formation and function of the mammary tree. The multipotent cap cell is capable of synthesizing laminin, hyuronate, and S-GAGs (33). However, only hyuronate is confined to the region of the end bud tip (33). During the establishment of the open ductal arrangement in the nonpregnant rodent mammary gland, collagen fibrillogenesis plays a significant role in the maturation of the mammary tree (33). As a part of this process, stromal synthesis of S-GAGs forms a unique concentration gradient around the end bud flank, which may act in an inhibitory manner to discourage other ducts from approaching the same area of growth, thereby channeling the direction of terminal end bud growth (33). Recently, HS-GAGs have been implicated in suppression of AR signaling (14, 34, 35). The NH₂-terminal hydrophilic region of the human AR protein contains a lysine-arginine-rich heparin-binding region adjacent to the COOH-terminal end of the EGF-like domain (14). *In vitro,* the mitogenic activity of AR can be modulated in an inhibitory fashion by the addition of exogenous heparin (14).

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**Fig. 8.** Summary of AR activity in the mouse mammary gland. All images represent the activity of the AR gene and its recombinant protein in the context of the intact normal, OVX, regressed, and serial transplanted mammary gland in vivo.
Furthermore, heparin is structurally related to heparan sulfate (36). Piekorn et al. (35) recently found that in mouse keratinocytes, soluble heparin-like GAGs can inhibit the mitogenic activity of AR, whereas the membrane-bound S-GAGs may bind to the cell membrane receptor or matrix and may act as mediators in AR signaling. They also suggest that during the process of keratinocyte commitment to terminal differentiation, the expression of membrane-bound endoglycosidase may be up-regulated. This enzyme, in turn, then generates soluble heparan sulfate free chains by cleaving the membrane or matrix-bound HS-GAGs. The exogenous heparan sulfate free chains can compete with S-GAGs for AR binding and disrupt the AR signaling pathway, which results in the cessation of keratinocyte growth (35). In support of this hypothesis, Johnson and Wong (14) suggest that in mammary epithelial cells, HS-GAGs maybe covalently linked to membrane proteins, such as syndecans, that stabilize the mitogenic signaling pathway between AR and the EGFR (14).

In this regard, we speculate that in the context of the normal, intact, and OVX mammary gland, the AR signaling pathway may be mediated through these mechanisms. This speculation can be supported by the recent observations in the juvenile C57 BL6 female mammary gland. We have demonstrated that in the quiescent rudimental mammary ducts of 3-week-old C57 BL6 female mice, AR protein expression is limited to the epithelial component. However, in the 4–8-week-old actively growing mammary gland, AR protein expression was detected in the adjacent stroma ahead of the migrating TEBs and in the stroma flanking the region of subtending duct (7). Given this scenario, we suspect that with the concurrent overexpression of AR, the appearance of abnormal developmental structures (Fig. 6, curved arrow and open arrow) may have been derived by, but not solely attributed to, a modified multipotent cap cell that is responding to: (a) an abundant source of AR protein complexed with HS-GAGs that maximize a constitutive EGFR autocrine signaling loop anchored on its surface at the end bud tip; and (b) the effect of abundant AR protein during the normal course of collagen fibrillogenesis that allows for an unfavorable S-GAG:AR ratio in the S-GAG gradient in the end bud flank, resulting in dysregulated ductal growth. To clarify these hypotheses, we are currently conducting AR and EGFR in situ hybridization, immunofluorescence, and S-GAG staining.

**Does the Activity of AR Share EGF-like Characteristics?** We suggest that the biological activity and patterns of immunolocalization of the AR protein in the developing mammary ductal compartments resemble EGF more than TGF-α. Using the slow-release Elvax pellet technique, recombinant AR stimulated a mitogenic and ductal growth pattern quite similar to that which is induced by recombinant EGF. However, when AR is overexpressed in normal mouse mammary epithelium in vivo, the resultant epithelium resembles that of TGF-α transgenic tissue. AR transgenic mammary tissue consistently developed a dense network of branching ducts similarly found in TGF-α transgenic pubescent mammary glands (21). Likewise, both TGF-α and AR have the capacity to stimulate lobular alveolar development in ovarian-compromised mammary tissue (6). Currently, there is no evidence of the direct consequence of EGF transgene in the context of the rodent mammary gland (25). In vitro, AR has the capacity to act as a potent epithelial mitogen for several human breast cancer cell lines (25, 37). Under growth factor-depleted conditions, AR can replace the growth-promoting effects of EGF (37). In addition, the proliferation of human immortalized mammary epithelial cells may rely in part upon the production of endogenous AR. Recently, our group has shown that the growth of nontransformed, immortalized mammary epithelial cell line 184 A1N4, which expresses AR mRNA, can be growth inhibited by specific antisense oligonucleotides in the presence of exogenous EGF. However, a combination of endogenous antisense TGF-α mRNA expression and addition of antisense AR oligonucleotides did not lead to a further cessation in growth when compared to the inhibitory effects of an anti-EGFR neutralizing antibody alone. This may suggest that in nontransformed mammary epithelial cells, additional endogenous non-EGF-related mitogens may act to stimulate growth (37).

What is the consequence of AR expression? In the context of the mammary gland, the biological activity of AR is equivalent to EGF, and the transgene effect parallels that of TGF-α. In the paradigm of mammary transformation, we hypothesize that the activity of AR in mammary epithelial stem cell progenitors facilitates maturation arrest and allows these cells to enter into the malignant pathway via EGF independence. This hypothesis has been conceptualized by Medina et al. (26), who suggest that some molecular alterations that contribute to the development of mammary preneoplasias and neoplasias may be EGF-linked. The relationship between ovarian dependence and EGF dependence (preneoplasia I) and EGF independence and tumorigenic potential (preneoplasia II) may indicate that the participants in the EGF pathway (i.e., AR) play important roles in the generation of mammary preneoplasia which have measurable tumorigenic potential (26). Interestingly, we have observed previously that the AR gene is expressed in mammary tumors generated from TGF-α, neu, int-3, polyoma middle T antigen, and SV40 large T antigen transgenic strains (20). Of the three growth factor/receptor-related transgenic mammary tumors (TGF-α, neu, and int-3) the growth of both TGF-α and int-3 transgenic mammary tumors cell lines is EGF independent in vitro. The AR protein in these tumors undergoes a temporal increase and preferential processing in a strain-specific fashion. In the current study, we could not detect preferential processing but did identify a 6.5-fold increase in two AR isoforms from AR-transduced outgrowths compared to controls. It is very likely in this experimental model that secondary genetic germ-line events contribute to tumorigenesis and to preferential processing of the AR gene product. Additional studies that approach the biochemical effects of the AR gene in vivo and in vitro should shed further insights into the activity of this gene. Thus far, our combined approach has provided a unique and in-depth analysis into the AR gene and its biological activity. Our study provides additional evidence for the importance of the AR gene and

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*G. Smith, personal communication.*
the AR protein and its contribution during various stages of mammary development and cancer. We suggest that the data accrued here can potentially lead toward the development of diagnostic and therapeutic strategies.

Materials and Methods

Animals and Pellet/Transplant Surgery. A total of 75 (50 C57 B16 and 25 FVB/N; Charles River Laboratories) pathogen-free mice O VX at 5 weeks of age were used in the Elvax slow-release pellet experiment. These inbred mice were also housed for 2.5 months prior to pellet implantation to eliminate the possibility of systemic sex steroid effects on mammary ductal morphogenesis. Before the experiment, a small L-shaped incision was placed on the abdominal wall of the mouse, exposing the fourth inguinal and contralateral mammary glands (23). Next, a small pocket within the mammary tissue was placed by small forceps. An Elvax pellet containing growth factor or BSA (control) was placed within the pocket, and the incision was closed with wound clips. For each experiment (repeated three times), a total of 10 O VX mice received AR Elvax pellets (5 µg each), 10 O VX mice received EGF (5 µg each), and 5 O VX mice received BSA Elvax pellets (5 µg each).

In transplantation experiments that used retrovirally transduced AR mammary primary cultures, pNO4 (empty vector)-transduced mammary primary cultures and control nontransduced mammary primary cultures were a total of 75 3-week-old virgin female C57 B16 mice were used. A total of 15 13-week-old hormonally intact female C57 B16 mice were used to derive primary mammary epithelial cultures. In each experiment (repeated three times), a total of 10 mice received AR-transduced primary cells, 10 mice received pNO4 (empty vector)-transduced primary cells, and 5 mice received control nontransduced primary cells.

In the second set of experiments that used serially transplanted outgrowths into syngeneic hosts, a total of 15 mice were used in this study. Briefly, five hormonally intact 3-week-old female mice, four 5-week-old O VX mice, and four 3-month-old fertile female mice received a pooled fragment (2 × 10⁵ cells) of AR hyperplastic outgrowths from living donors (a total of three). In all experiments, the fat pads of these mice were cleared of the host’s epithelial rudiment. Fragments were allowed to repopulate the hosts mammary epithelium for 4–12 weeks or 12 weeks for mice that were eventually mated and sacrificed at 5 and 28 days, respectively. In all experiments, animals were supplied with food and water ad libitum and were housed under a 12-h light/12-h dark cycle. Upon surgery or completion of the experiment, animals were either anesthetized with metofane or euthanized with CO₂ and metofane.

Implants. Ethylene acetate copolymer (Elvax) was gift from the DuPont Chemical Co. AR was a gift from Dr. Stuart Thompson (Belfax Biosciences, Concord, CA). EGF was purchased from Collaborative Research (Waltham, MA). Briefly, 20–25 mg of BSA and 100 µg of AR and EGF were dispersed in 125 µl of Elvax dissolved in dichloromethane (20%/w/v). The mixture was quick frozen, dried, and cut to weight (1.0 mg = 5 µg of growth factor or BSA). The pellets were surgically implanted into the fourth mammary gland, and the BSA pellet alone was implanted in the contralateral no. 4 mammary gland.

Retroviral Vectors. An 850-bp human AR fragment that contains the coding sequence for AR was derived from a full-length 1.4-kb human AR cDNA as described previously (15). This fragment was then introduced in the 5′ to 3′ orientation into the Moloney sarcoma virus-long terminal repeat-derived pNO4 recombinant plasmids at the 3′ end of the cytomegalovirus viral promoter (37). Transfection of the recombinant 850-bp AR sense plasmid was then carried out as described previously into the PA 317 amphotrophic packaging cell line to produce amphotrophic, replication-defective retrovirus stocks for AR (13, 37). Virus-containing supernatants were obtained from mass-transfected G418-resistant PA 317 cells. These supernatants were then screened for helper virus and found to be negative (38). Primary cells were infected with PA 317 retroviral vector containing supernatants (1 ml) that had been previously titrated on NIH 3T3 cells at 1.5 × 10⁶ neo/macroglobulin colonies/ml. Primary mammary epithelial cell cultures in medium containing 4 mg/ml DEAE dextran. Primary cells were later maintained in G418 for an additional 7 days before transplantation.

Primary Cultures. Primary mouse mammary epithelial cultures were obtained and transplanted as described previously (7). Briefly, the mammary glands from a total of 15 13-week-old hormonally intact female C57 B16 mice were used to derive primary mammary epithelial cultures. Isolated cultures used for infection were allowed to grow in Improved Modified Eagle’s Medium supplemented with 10% FBS, 10 µg/ml bovine insulin, 5 µg/ml hydrocortisone, and 10 ng/ml EGF at 37°C and 5% CO₂ for 7 days before transplanting 1 × 10⁶ cells into the cleared fat pads of intact 3-week-old syngeneic hosts.

Histology. Transplanted or implanted (Elvax slow-release pellets) mammary glands were excised and fixed overnight in 4% formalin. Whole-mount analysis were prepared by first defatting the tissue in acetone, then hydration through alcohols, stained with Gill's hematoxylin, and dehydrated and mounted for photography. Whole glands were then embedded in paraffin and sectioned for immunohistochemical analysis (Brdu/Ur labeling) or morphometric characteristics by staining with H&E. Morphometric characteristics are measurements that calculate ductal width, length and degree of branching, and secretory lobules. Briefly, for the degree of branching and secretory lobules present, a total of 20 random fields from five AR-transduced and five control nontransduced outgrowths were examined under tight microscopic. For morphometric measurements, a total of 20 random fields from five AR-transduced outgrowths and five control nontransduced outgrowths were photographed and exposed on 5 × 7 Kodak prints and then standardized in millimeters for the origin length of the image. Finally, the distances and widths of mammary ducts were calculated in micrometers.

Scoring of Labeled Nuclei. Briefly, 50 C57 B16 5-week-old O VX mice that were implanted and treated with either AR, EGF, or BSA Elvax pellets for 5 days were then injected with BrdUrd (100 mg/kg; Sigma Chemical Co.) i.p. into the lower right quadrant of the abdomen 2 h prior to sacrifice. Mammary glands were excised and embedded in paraffin and sectioned for immunocytochemistry. Sections were cut through xylene and graded alcohols, and endogenous peroxidase activity was inactivated with 0.03% H₂O₂ in methanol. Nonspecific binding was blocked with 5% normal goat serum, after which the sections were incubated for 12 h with 1 µg/ml of mouse anti-BrdUrd antibody (Sigma Chemical Co.). A section of duode- num was used as a control to confirm systemic delivery of BrdUrd to tissues. Sections were then treated with 1:1000 dilution of rat antirat antibody (Vestacant) and visualized with avidin-biotin complex conjugated to horseradish peroxidase with 3'-3'-diaminobenzidine-4-HCl as the chromogen (Vestacant Elite ABC kit; Vector Laboratories, Burlingame, CA). The slides were then lightly counterstained with Gill's hematoxylin or carmine aluminum. Random fields surrounding the periphery of the pellet were used for BrdUrd scoring. A total of 20 fields scored in 10 sections from each treatment group contained more than 100 cells/field. Percentages of labeled cells were calculated by dividing the number of nuclei in each field by the total number of nuclei counted (8). Mammary epithelial cells that had BrdUrd incorporation were identified by dark brown to tan over purple (negative) pigment.

Western Analysis. Primary cultures or the fourth inguinal mammary gland tissue was homogenized in 1 ml of hypotonic buffer (20 mM HEPES pH 7.4, 1 mM EDTA, 1 mM MgCl₂, 1 mM PMSF, 1% NP40, and 20 µg/ml aprotinin; Ref. 13). Samples (50 µg) were boiled and resolved on a 4/15% SDS-PAGE gel. AR was detected by an affinity-purified anti-AR antibody (7). Equivalent loading of protein samples was observed by staining a parallel gel with Coomassie Blue. Specificity was confirmed by pre-absorbing AR antibody against a 5-fold excess of the synthetic peptide 26-44.

Southern Blot Analysis. Genomic DNA was extracted from transplanted outgrowths using the proteinase K-pheno extraction method (39). The DNA was then digested with HindIII/XhoI, separated by electrophoresis in 1% agarose, and transferred to nylon filters. A 2.7-kb probe containing the neomycin gene-cytomegalovirus promoter region of pNO4 complimentary DNA plasmid was generated by HindIII/XhoI restriction digestion. Southern hybridization was carried out at 84°C in hybridization buffer from Clone Tech (Palo Alto, CA) and 15 ng/ml probe labeled with [γ-³²P] ATP (Amersham Corp.) by random priming (Boehringer Mannheim, Indianapolis, IN). After overnight hybridization, the blots were washed in 0.2 x SSC-1% SDS at 65°C for 20 min and then washed with 0.1 x SSC-0.1 SDS at 65°C for 5 min. The blots were
subjected to autoradiography using Kodak films at −70°C with an intensifying screen.

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References


