Reconstruction of functionally normal and malignant human breast tissues in mice

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The study of normal breast epithelial morphogenesis and carcinogenesis *in vivo* has largely used rodent models. Efforts at studying mammary morphogenesis and cancer with xenotransplanted human epithelial cells have failed to recapitulate the full extent of development seen in the human breast. We have developed an orthotopic xenograft model in which both the stromal and epithelial components of the reconstructed mammary gland are of human origin. Genetic modification of human stromal cells before the implantation of ostensibly normal human mammary epithelial cells resulted in the outgrowth of benign and malignant lesions. This experimental model allows for studies of human epithelial morphogenesis and differentiation *in vivo* and underscores the critical role of heterotypic interactions in human breast development and carcinogenesis.

The mammary gland is an organ that undergoes most of its development postnatally. This development requires a complex interplay of systemic hormones and local growth factors.

Analyses of the complex heterotypic interactions between epithelium and stroma in mouse models of mammary development and carcinogenesis have been performed through the widely used cleared fat-pad transplantation system (1). This technique takes advantage of the fact that the mouse mammary gland is not fully developed at 3 weeks of age, making it possible to excise the rudimentary mouse mammary epithelium from the fat pad; this yields a "cleared" fat pad devoid of any epithelium. Subsequent engraftment of mammary epithelial cells (MECs) before puberty yields an anatomically normal functional mammary gland that lacks only connections with the nipple. The successful outgrowth of the engrafted MECs indicates that the systemic hormones present during puberty, together with the stroma of the cleared fat pad, provide all of the signals that are required for normal epithelial morphogenesis.

Attempts to recapitulate human breast epithelial morphogenesis by introducing human MECs into the cleared mammary fat pads of mice have not yielded similar results. Unlike their murine counterparts, the introduced human MECs fail to colonize these fat pads and thus do not develop into ductal and lobular structures (2, 3). To circumvent this issue, researchers have attempted to recreate functional tissues by transplanting normal breast tissue fragments or collagen-embedded dissociated human MECs either s.c. or beneath the renal capsule of immunocompromised nude mice (2–10). Although the tissue fragments and dissociated cells survive and respond to hormone stimulation, no outgrowth or expansion of the transplanted tissues has been observed.

For these reasons, we sought to develop a new protocol for the establishment of human mammary stroma within the mouse mammary fat pad, anticipating that the resulting stroma would provide the proper environment for the development of human mammary epithelium.

Materials and Methods

Plasmids, Tissue Culture, and Cells. All human breast tissue procurement for these experiments was obtained in compliance with laws and institutional guidelines approved by the Institutional Review Board committee from Brigham and Women's Hospital and the Committee on the Use of Humans as Experimental Subjects at Massachusetts Institute of Technology. Tissues were obtained from discarded material from patients undergoing elective reduction mammoplasty between the ages of 29 and 37 years.

Primary human breast fibroblasts and organoids were isolated from reduction mammoplasty tissue as described (11, 12). Briefly, breast tissue was chopped into 1-mm cubes and dissociated for 12 h in a solution of collagenase (3 mg/ml) and hyaluronidase (600 μg/ml). Fibroblasts were separated from organoid–epithelium by differential centrifugation. Fibroblast cells were grown in DMEM supplemented with 10% calf serum and cultured for $<$14 days to expand the cells. Organoids were frozen in cell-freeze medium until surgery.

To generate the breast fibroblasts used for "humanization" of mammary fat pads termed reduction mammary fibroblasts immortalized with human telomere and GFP (RMF/EG), primary breast fibroblasts. Polyclonal populations of fibroblasts were naturally selected through serial passages until a pure GFP-positive population of cells emerged as examine by fluorescence-activated cell sorter analysis.

These fibroblasts were subsequently subjected to retroviral infections of hepatocyte growth factor or transforming growth factor β with drug selection used to purify polyclonal-infected populations after each infection as described (13). Drug selection of infected fibroblasts was performed with 700 μg/ml zeocin.

Retroviral constructs were generated to encode for the catalytic subunit of human telomerase (hTERT) and the GFP (pLUC-hTERT). Retroviral constructs for hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β) were generated by cloning the full length cDNA of HGF (kindly provided by G. Vande Woude) or TGF-β (kindly provided by R. Derynck, University of California, San Francisco) into the pBABE-zeocin vector system (Clontech).

Animals and Surgery. A colony of immunocompromised NOD/SCID mice was maintained in-house under aseptic sterile conditions. Mice were administered autoclaved food and water ad libitum. Surgeries were performed under sterile conditions, and animals received antibiotics in the drinking water up to 2 weeks after all surgical procedures.

Three-week-old female mice were anesthetized and the mammary epithelium was removed from the no. 4 inguinal mammary

Abbreviations: MEC, mammary epithelial cell; HGF, hepatocyte growth factor; PCNA, proliferating cell nuclear antigen; FISH, fluorescence *in situ* hybridization; TDLU, terminal ductal lobular units; TGF-β, transforming growth factor β; RMF/EG, reduction mammary fibroblasts immortalized with human telomere and GFP.

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glands of NOD/SCID mice. Two weeks later, 2.5×10^5 unirradiated RMF/EG fibroblasts and 2.5×10^5 irradiated (4 Gy) fibroblasts were injected into the cleared fat pads 24 h after irradiation. Two weeks after the introduction of the human stromal cells, human breast epithelial organoids and primary fibroblasts were injected into the humanized site. For this, organoid preparations were thawed, and 20–25 organoids were mixed with 2.5×10^5 primary fibroblasts and resuspended in 35 μl of a 3:1 Collagen I (Upstate Biotechnology, Lake Placid, NY): Phenol-red free Matrigel (Becton Dickinson) mixture and injected into the humanized mammary glands.

Whole Mounts and Immunohistochemistry. For murine whole-mount analysis, fresh mammary tissue was flattened and fixed in carnoy's solution (ethanol, glacial acetic acid, and chloroform) and subsequently stained with carmine alum (carmine, AlKSO4). Human breast tissue was analyzed by whole mount by using hematoxylin. Briefly, fresh tissue obtained from reduction mammoplasty was sliced into 2 cm $\times$ 1 mm $\times$ 1 mm fragments and fixed in 10% neutral buffered formalin. Tissues were defatted through graded organics and alcohols and stained in hematoxylin.

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues. Five-micron sections were deparaffinized, rehydrated through graded alcohols, and subjected to antigen retrieval for immunohistochemistry. Sections were incubated in mouse monoclonal antibodies against smooth muscle actin (1:50, NovoCastra, Newcastle, U.K.), human-specific vimentin (1:50, NovoCastra) estrogen receptor (DAKO) progesterone receptor (DAKO), keratin 19 (NovoCastra), proliferating cell nuclear antigen (PCNA) (DAKO), p53 (Santa Cruz Biotechnology), human β-casein (gift from Charles Streuli, University of Manchester, Manchester, U.K.). Immunocomplexes were visualized by the ABC peroxidase method (Vector Laboratories, Burlingame, CA), and sections were counterstained with hematoxylin or methyl green.

Genomic Fluorescence *in Situ* Hybridization (FISH). Tissue sections were assayed for the presence of mouse, and human cells by using FISH with species-specific genomic probes as described (14). Briefly, 5-μm sections were deparaffinized in xylene, dehydrated in ethanol, treated with NaSCN, RNase, pepsin, and finally HCl, and subsequently dehydrated in 70%, 85%, and 100% ethanol series. Sections were denatured and hybridized with probes against CY3-d-UTP-labeled mouse Cot 1 DNA (GIBCO), or FITC-12-d-UTP-labeled human genomic DNA extracted from lymphocytes. Probe sizes ranged between 300 and 2,000 bp.

DNA Fingerprinting. Genomic DNA was isolated from frozen organoid preparations or five 5-μm sections of paraffin embedded normal or tumor outgrowth tissue from xenografted material and subjected to DNA fingerprinting as previously described (15).

Results

Generation of "Humanized" Mammary Fat Pads. We initially engrafted human MECs into cleared mouse mammary fat pads. In doing so, we confirmed earlier reports (2), indicating that human epithelial cells are unable to establish themselves in the murine mammary stroma and to participate in normal mammary ductal morphogenesis. The failure of human MECs to properly colonize the mouse mammary fat pad suggested the need to establish human mammary stromal cells in fat pads before implantation of the epithelial cells. Accordingly, we developed a line of GFP-labeled human telomerase-immortalized human mammary stromal fibroblasts; these cells had previously been prepared from a reduction mammoplasty. These fibroblasts (hereafter termed RMF/EG) are diploid, ostensibly normal, and are nontumorigenic, as they do not form colonies in soft agar and do not form tumors in immunocompromised mice when examined after 9 mo (data not shown).

When the RMF/EG fibroblasts were injected into the mammary glands of unirradiated NOD/SCID mice, the cells survived, but little proliferation or expansion of the introduced cells was observed (data not shown). We therefore sought to facilitate their engraftment and subsequent proliferation by exploiting observations made by others that irradiated fibroblasts undergo a physiologic activation that results in the expression of proteases, matrix proteins and a variety of growth factors (16, 17). Accordingly, we irradiated 2.5×10^5 immortalized RMF/EG fibroblasts with 4 Gy of radiation and injected these together with an equal number of nonirradiated RMF/EG fibroblasts into cleared mouse mammary fat pads of NOD/SCID mice. This was done with the hope that the irradiated fibroblasts would enable the engraftment and invasion of the coinjected nonirradiated cells in the mouse stromal fat pad.

Mammary fat pads that had been injected with these cell mixtures were examined by carmine whole-mount analysis at various times between 2 and 8 weeks postinjection, and no detectable changes were observed. However, examination of the mammary fat pads for GFP expression at 4 and 8 weeks after injection clearly demonstrated sites of engraftment and expansion of the human breast fibroblasts within all of the inoculated fat pads, with 5–15% of the fat pad occupied with human stromal cells. (Fig. 1 *A* and *B*). Invasion of individual human fibroblasts into the mouse adipose

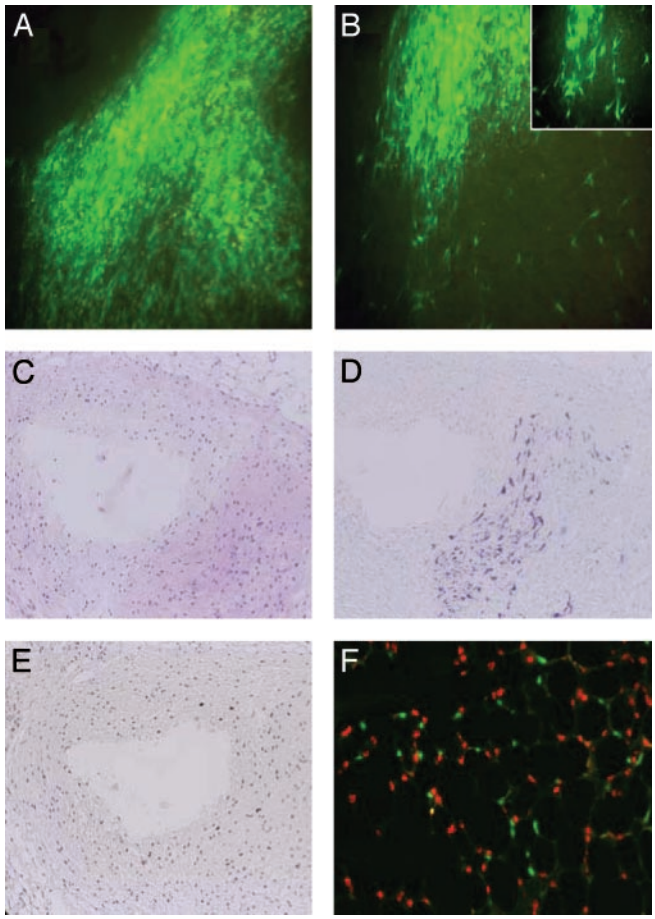

Fig. 1. GFP whole-mount and histological analysis of human–murine chimeric fat pads. (*A* and *B*) GFP whole-mount examination of cleared mouse mammary fat pads injected with immortalized human breast fibroblasts 4 weeks postinjection ($\times$10). Single GFP-positive cells can be seen infiltrating the adipose stroma (*B Inset*). (*C*) Hematoxylin/eosin-stained section of an area of fibroblastic cord growth within the cleared fat pad. (*D* and *E*) Serial sections of this region were examined by immunohistochemistry by using antibodies against human-specific vimentin (purple, *D*) or PCNA (brown, *E*). Genomic FISH was performed by using specific probes for mouse Cot I gene (red) and human genomic DNA (green) (*F*).

stroma could be observed at high magnification. In addition, large cords of GFP-labeled fibroblasts could also be detected by histology in some of the fat pads (Fig. 1*C*).

In these cases, the cords of cells were found to be comprised of proliferating fibroblasts expressing human-specific vimentin (Fig. 1 *D* and *E*) and PCNA. However, because the great majority of fat pads did not contain readily apparent cords of engrafted fibroblasts, it was difficult to confirm "humanization" by histology in most cases. Therefore, to address whether the RMF/EG fibroblasts had indeed survived and colonized the mouse mammary cleared fat pads, genomic FISH was performed on cleared mammary stromal tissues that did not contain histological evidence of stromal outgrowth at either 4 or 8 weeks after injection. A probe specific for mouse Cot1 DNA or for human genomic DNA (prepared by labeling unfractionated human DNA) was hybridized against sections of the humanized mouse mammary fat pads. Examination of these tissues revealed the existence of the human breast fibroblasts interspersed among murine adipose tissue, indicating that these human cells had indeed survived and integrated within the mouse mammary stroma (Fig. 1*F*).

Development of Human Breast Ducts in Chimeric Fat Pads. We sought to exploit these humanized fat pads as tissues into which we could subsequently introduce human MECs. Accordingly, we prepared human MEC organoids from histologically normal human reduction mammoplasty tissues. Organoids are clusters of human myoepithelial and luminal epithelial cells, each containing 100–1,000 cells that are no longer organized in structures resembling those in the normal human breast (18). Twenty to 25 organoids were mixed with 2.5×10^5 primary human breast fibroblasts that had also been recently isolated from reduction mammoplasty tissues. The mixed cell preparations were then introduced into the murine stromal fat pads that had been humanized 2 weeks earlier through the introduction of human stromal fibroblasts.

Eight weeks after the introduction of the organoids and admixed fibroblasts, the xenografted mammary glands were removed and subjected to whole-mount analysis. Three types of epithelial outgrowths were reproducibly observed among all of the xenografts. The relative frequencies of these ductal, lobular, and acinar structures varied from one set of donor organoids to another (Fig. 2). When the primary normal breast fibroblasts were not mixed with these organoids before engraftment into humanized fat pads, only stunted and deformed structures developed (data not shown).

The most frequently observed outgrowths were of acinar shape, consisting of spherical structures with hollow lumina. Less commonly observed were linear ductal outgrowths with little side branching (Fig. 2*B*). Ductal development occurred in the absence of additional ectopic estrogen or progesterone hormonal stimulation if the engraftment process was undertaken before the end of puberty. Similar to other observations with engrafted murine MECs, the frequency of human ductal elongation decreased (to <20%) when engraftment of the organoids and fibroblasts occurred after puberty in the recipients (data not shown). In addition to this ductal and acinar development, full human ductal–lobular development occurred when mice bearing these human grafts became pregnant (Fig. 2*C*).

Genomic FISH was performed on paraffin sections of the xenograft outgrowths to determine the origins of the epithelial and stromal cells in the xenografts (Fig. 3*A*). As anticipated, all of the epithelial structures were of human origin, and a significant proportion of the stroma was also of human origin. However, a substantial number of murine cells could also be found in the stroma. A vascular response was observed within the stroma surrounding the xenografts, as evidenced by the dilation of existing vessels and neovascularization that had apparently occurred as a part of normal epithelial development (Fig. 3 *B* and *C*).

Normal human mammary ducts are composed of luminal MECs separated from the basement membrane by an intervening layer of myoepithelial cells. Indeed, cells located on the basal side of the ducts formed within these grafts stained positive for smooth muscle actin and lacked expression of the estrogen receptor, indicating the presence of myoepithelial cells (Fig. 4*C*). Conversely, luminal cell-specific markers, such as cytokeratin 19, estrogen receptor, and E-cadherin, were expressed in the luminal cells of the ducts (Fig. 4 *C–E*). More than 10% of epithelial cells and 2% of the stromal compartment of the xenografts stained positive for PCNA (Fig. 4*F*). Because normal human breast tissue rarely contains proliferating stromal cells and <2% of epithelial cells proliferating at any given time during the menstrual cycle (19), this indicates that cells in the reconstructed breast tissue were actively proliferating *in vivo*.

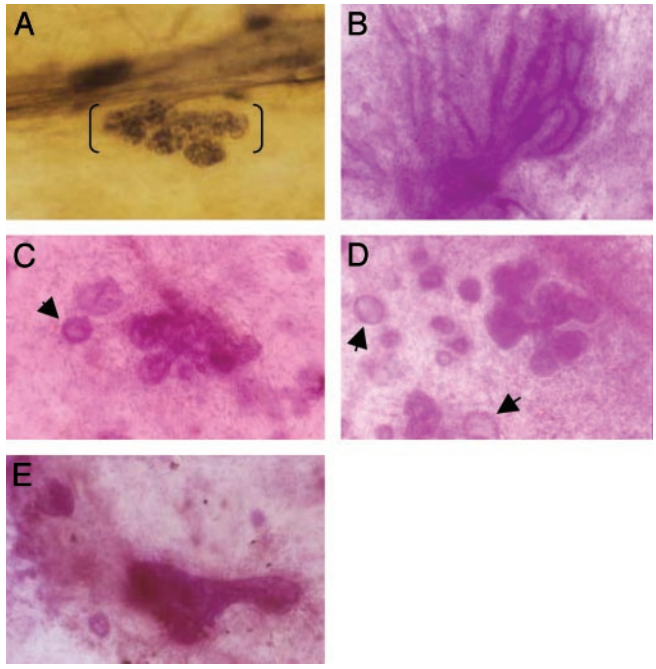

Fig. 2. Whole-mount analysis of breast tissue of human breast and xenografted outgrowths. Normal human breast tissue isolated from reduction mammoplasty was stained with hematoxylin and whole-mounted exhibits both ductal and TDLU (in brackets) structures (*A*). Carmine-stained whole-mount examination of outgrowth from xenografts in humanized fat pads contain both ductal (*B*) and TDLU structures (*C* and *D*). Arrows point out commonly observed acini structures with hollow lumina. TDLU formation occurred during murine puberty (*C*) or pregnancy (*D*). Some outgrowth displayed evidence of both ductal and TDLU-like growths (*E*).

Mice bearing xenografts were mated and allowed to develop until day 18 of pregnancy to determine whether full functional differentiation of the human breast tissue had been achieved. Indeed, we observed active lipid synthesis and secretion into the lumina of the human acini (Fig. 4*G*). Human β-casein expression could be detected both within the luminal MECs lining the acini and in the lumina of the human breast acini (Fig. 4*H*). Taken together, these various observations indicate that histologically normal functional human breast tissue can be constructed in mice, and that this outcome depends on the presence of human stromal cells. Moreover, the mouse mammary stromal fibroblasts that are present in the reconstructed stroma do not appear to impede this process. Furthermore, various stages of human mammary gland development can be recapitulated *in vivo*, including the production of milk.

Hyperplasic and Neoplastic Development of Engrafted Human MECs Due to Stromal Alterations. We speculated that the primary human mammary fibroblasts that were admixed to the human mammary organoids before engraftment might in some fashion affect the morphogenetic abilities of the human MECs in these organoids. To

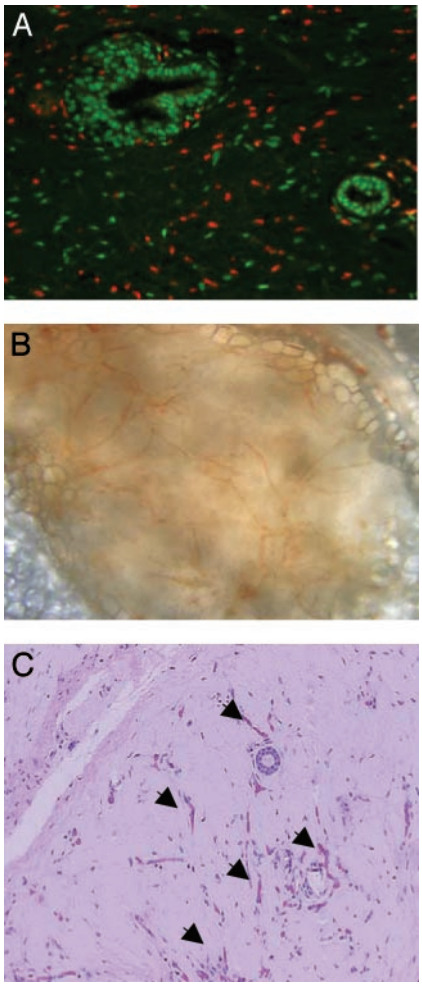

Fig. 3. Epithelial and stromal cell contribution in xenograft breast tissue. Genomic FISH was performed on sections from mice in which human breast epithelial cells were injected into the humanized fat pads along with normal primary breast fibroblasts (*A*). Human cells (green) comprise all of the epithelial cells in the xenografts, but the stroma is comprised of both human and mouse cells (red). A significant amount of angiogenesis is observed within the xenograft (*B*). White-light photomicrograph of the xenografted implanted region 8 weeks after surgery displays significant infiltration of blood vessels. (*C*) Hematoxylin/eosin-stained section of a region of the xenograft reveals many capillaries (arrows) within the stroma indicative of neovascularization.

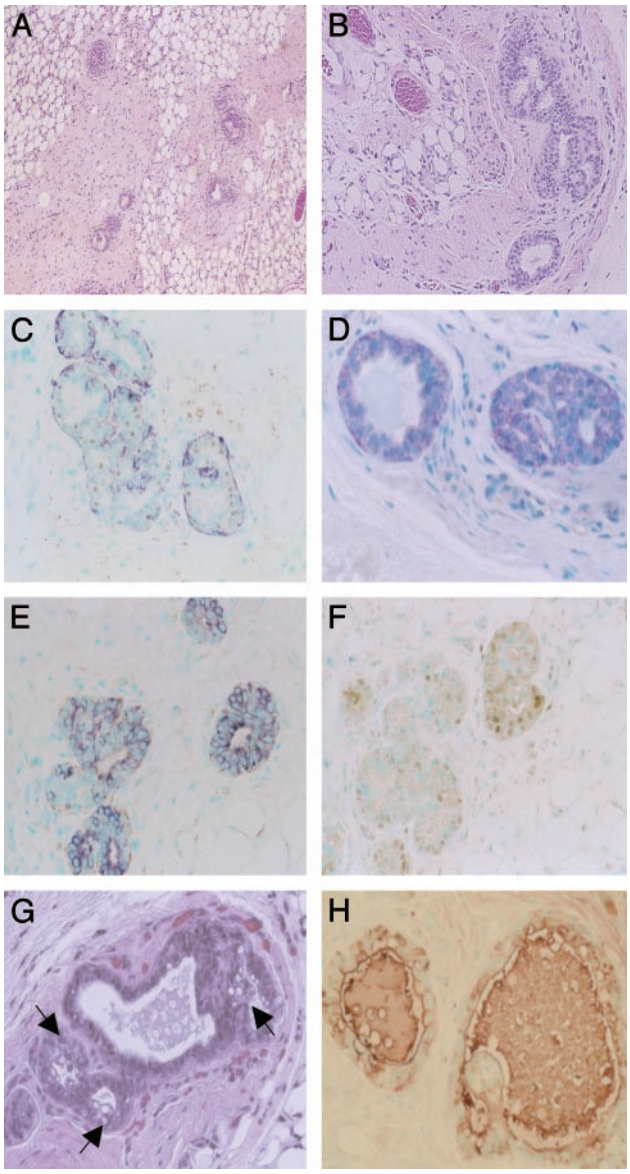

Fig. 4. Histological and molecular analysis of xenograft breast tissues. Paraffin sections of breast xenograft ductal outgrowths were stained with hematoxylin/eosin (*A* and *B*). Double labeling of breast markers ductal structures (*C*). Myoepithelial cell-specific expression of smooth-muscle actin (purple) is not detected in cells expressing the estrogen receptor (brown). Immunohistochemistry was performed on serial sections with antibodies specific for PCNA (*D*) to demonstrate the proliferative state of the outgrowth. Brown nuclei can be detected in both epithelial and stromal cells. Luminal-specific expression of cytokeratin 19 is detected in ER expressing cells (*E*), and E-cadherin expression is detected on the membrane of luminal epithelial cells (*F*). Paraffin sections of fully differentiated breast xenograft ductal outgrowths in pregnant mice were stained with hematoxylin and eosin (*G*). Milk protein and fat droplets are seen (arrows) in the differentiated epithelial cells under the control of the hormones of pregnancy. Immunohistochemistry was performed on these sections with antibodies against human-specific β-casein milk protein (*H*). β-Casein expression is detected in secretory epithelial cells as well as in the luminal space of the alveoli.

address this possibility, we introduced 10 human mammary epithelial organoid preparations isolated from 10 different mammoplasty patients into humanized stromal fat pads either with or without prior addition of primary human mammary stromal fibroblasts. In all cases, histological examination of the mammoplasty specimens had previously revealed no pathological abnormalities (A.R., unpublished observations).

In the ≈70 coengraftments with normal stromal fibroblasts, we observed various normal epithelial outgrowths with all 10 organoid preparations. These outgrowths were very similar to those observed in the initial experiment. Different outcomes were seen, however, when primary fibroblasts were not mixed with the organoids before implantation into humanized mammary stroma. Implantation of 7 of the 10 organoid preparations resulted in the formation of normal mammary epithelial structures, albeit with somewhat stunted development (data not shown). In contrast, after implanting ostensibly normal organoids from the remaining three women, we observed abnormal human mammary epithelial structures (Fig. 5 *A* and *B*). These structures are similar to commonly observed benign human breast proliferations, specifically hyperplastic ductal epithelia. Because we had never observed these hyperplasias in coengraftments with normal stromal fibroblasts, we concluded that normal primary fibroblasts are capable of suppressing hyperplastic growths that might be generated by preexisting, marginally abnormal MECs present in the mammoplasty specimens.

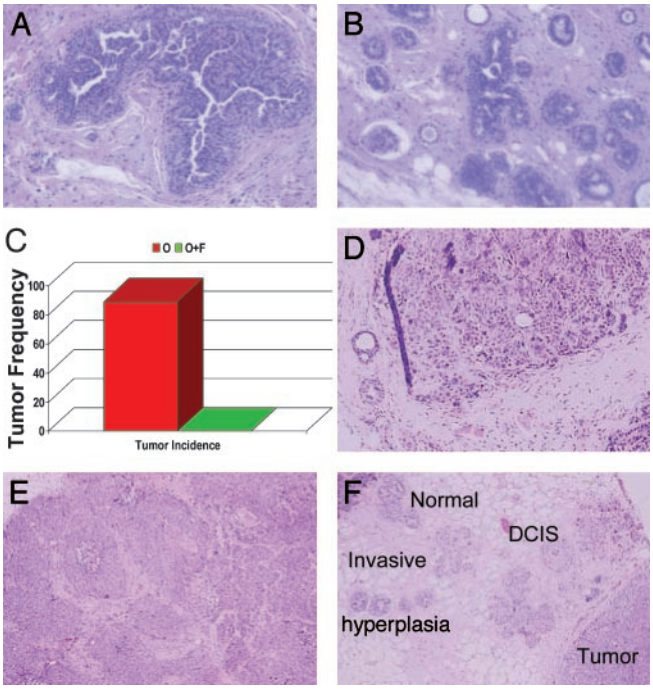

Fig. 5. Hyperplastic and tumorigenic outgrowths derived from reduction mammoplasty epithelium. Hematoxylin/eosin-stained sections of hyperplasias that developed from normal organoids in the absence of normal admixed fibroblasts (*A* and *B*). (*C*) Frequency of tumor formation when reduction mammoplasty organoids were introduced directly into humanized stroma overexpressing TGF-β or HGF (O) or when the organoids were coinjected with normal primary human breast fibroblasts (O + F). Histology of the tumors arising in glands humanized with growth factor expressing stroma: stroma that overexpresses either HGF (*D*) or TGF-β1 (*E*). Histology of outgrowths of various presumed stages of human breast cancer detected in the same field. Normal ductal tissue (normal), benign hyperplastic ducts (hyperplasia), *in situ* ductal cancer (DCIS), and invasive carcinoma (invasive) (*F*).

We speculated that we might reveal and highlight further differences between different preparations of organoids if we perturbed the stromal environment of the engrafted human organoids. Consequently, we modified the humanized stroma by forcing the RMF/EG fibroblasts to express either human HGF or human TGF-β1 before using them to humanize the stroma. In mouse mammary glands, overexpression of HGF is known to result in increased branching of the mammary ducts and in a generalized hyperproliferation of the epithelium (20). In contrast, TGF-β overexpression inhibits proliferation of MECs (21) but promotes alterations in the stroma, including the conversion of fibroblasts to myofibroblasts, the induction of neoangiogenesis, and other responses associated with wound healing (22, 23). Both of these growth factors are known to be synthesized by mammary stromal cells and are found to be overexpressed in the stroma of human breast cancers (24, 25).

Cleared mammary fat pads were humanized with RMF/EG fibroblasts that overexpress either HGF, TGF-β, or a mixture of both types of cells. Subsequently, human mammary epithelial organoids prepared from the 10 previously used reduction mammoplasty samples either were introduced directly into these growth factor-enriched fat pads or were mixed with normal primary stromal fibroblasts before implantation into the growth factor-enriched fat pads (Fig. 5). When mixed with normal primary fibroblasts, we observed normal acini, terminal ductal lobular units (TDLU), and even some ductal outgrowths from the organoids obtained from all 10 patient samples; these structures were very similar to those observed previously when these cells were implanted in humanized stroma that had not been engineered to release either of these growth factors (data not shown).

When the 10 organoid preparations were implanted directly into the growth factor-enriched stromal environment (and thus without prior admixture of normal stromal fibroblasts), 9 of the 10 preparations yielded poorly developed stunted ductal structures. Surprisingly, this was the case for the three samples that had yielded ductal hyperplasias in the previous experiment.

One of the 10 reduction mammoplasty specimens behaved quite differently, however. In this particular case, as described above, cleared fat pads had been humanized with fibroblasts that overexpressed HGF (12 mammary fat pads), TGF-β (eight mammary fat pads), or a mixture of both types of growth factor-overexpressing fibroblasts (16 mammary fat pads). When primary human breast fibroblasts were mixed with the organoids from this patient before engraftment into growth factor-enriched humanized fat pads, normal outgrowths were seen (Fig. 5*C*).

However, when the organoids from this patient were introduced directly into the growth factor-enriched humanized stroma (six HGF mammary fat pads, four TGF-β mammary glands, and eight HGF + TGF-β mammary glands) without admixed primary human stromal fibroblasts, the engrafted MECs developed into growths that closely resembled human ductal carcinomas, including both comedo- and basal-type carcinomas (Fig. 5 *D* and *E*). Indeed, carcinomatous growths were seen in all 16 mammary glands (18 less 2, because one TGF-β-humanized animal died prematurely). These tumors were invasive, and some were poorly differentiated. Interestingly, in one engrafted mammary gland derived from organoids of this patient, in which the stroma had been humanized with HGF-overexpressing fibroblasts, all of the presumed stages of human breast cancer progression, including hyperplastic ducts, carcinomas *in situ*, and even invasive carcinomas, could be observed in a single microscope field (Fig. 5*F*). These tumors were subjected to various immunohistological analyses that confirmed their transformed and invasive phenotypes (see supporting information). There were no significant differences in the histology of the tumors that developed in the fat pads humanized with TGF-β alone compared to the HGF or the TGF-β plus HGF fibroblasts. In addition, all of the tumors were confirmed to be of human origin, as ascertained by genomic FISH (data not shown).

In this experiment, 16 of 18 mammary gland grafts derived from this preparation of mammoplasty organoids yielded hyperplastic and neoplastic outgrowths. However, when we attempted to reproduce these histopathological abnormalities using organoids prepared from the same patient's mammoplasty sample (from a different region of the original mammoplasty tissue sample), we were unable to do so. We attribute this to interregional differences in the biology of various cell populations that preexisted within this breast tissue before mammoplasty. DNA fingerprinting was used to confirm that the epithelial preparation that gave rise to these neoplasms indeed derived from the presumed reduction mammoplasty biopsy (see supporting information, which is published on the PNAS web site).

Taken together, these observations indicate that an altered stromal environment can promote human breast cancer formation by abnormal cells that are present in the normal human breast and elude detection by normal histopathological screening. We speculate that these abnormal cells had already undergone one or several of the steps of tumor progression before their excision through mammoplasty. These histological abnormalities, evaluated independently by two breast cancer pathologists, left us with a single clear conclusion that the mammary gland reconstruction procedure, as described here, can allow the formation of many of the steps of human breast cancer progression, including ductal hyperplasia, carcinoma *in situ*, and invasive ductal carcinoma.

Discussion

In attempts to recreate breast tissues in mice, previous studies have attempted to introduce tissue fragments or dissociated MECs into the cleared mouse mammary fat pads of immunocompromised mice (2, 3). These attempts were unsuccessful in recreating functional, properly differentiated breast tissues, presumably due to the inadequate stromal environment provided by the mouse mammary fat pad.

The success of the irradiated fibroblasts in enabling cointroduced unirradiated fibroblasts to survive and colonize the mammary gland appears to be related to the highly activated microenvironment created by these irradiated cells; this microenvironment is characterized by remodeling of the extracellular matrix proteins of the adipose stroma, including increased collagen synthesis and activation of TGF-β (22, 23, 26). Due to the high rate of cell death associated with irradiation of fibroblasts, we restricted the irradiation process to only a portion of the fibroblast population before engraftment, hoping that the activated irradiated fibroblasts would assist in the survival and engraftment of the nonirradiated fibroblasts within the mammary fat pad of mice, an outcome that was indeed observed.

It is worth noting that, as shown here, the systemic hormonal environment of NOD/SCID mice during puberty or pregnancy is sufficient to promote human MEC proliferation and lactogenic differentiation. The use of immunocompromised NOD/SCID mice rather than nude mice in these experiments may account for the fact that additional ectopic estrogen stimulation was not required for MEC proliferation *in vivo*, as has been reported previously (9). Unlike nude mice, whose reproductive system and thus hormonal environment are compromised (27), NOD/SCID female mice are fertile, with a fully functional reproductive system. This indicates that the previously observed graft–host interspecies incompatibilities derive from paracrine heterotypic interactions rather than from incompatibilities in the systemic hormonal environment.

We have also demonstrated that histopathologically normal human breast tissues from reduction mammoplasty samples may not behave normally in the absence of a normal stromal environment. We observed this in three patient samples that gave rise to hyperplasias in the absence of admixed fibroblasts and, more strikingly, in one patient sample that gave rise to the various stages of breast cancer progression after implantation without admixed fibroblasts into growth factor-activated humanized stroma.

Given the rapidity with which these lesions and tumors formed (<8 weeks) and the minimal time they spent outside living tissue (<1 day), we consider it highly unlikely that the human MECs acquired additional genetic alterations after being removed from human breasts and introduced into the immunocompromised mice. We therefore conclude that these human epithelial cells had already undergone one or several premalignant changes before their implantation into mice. These abnormal cells were not initially detected by routine histopathological analyses of the donor mammoplasty specimens, either because they were present in only a small proportion of the breast tissue or because their abnormal phenotypes could not be realized in the context of a normal mammary stromal environment.

The notion that occult preneoplastic or neoplastic cells exist in breast tissues from young and middle-aged women who have not been diagnosed for breast cancer or in tissues obtained from reduction mammoplasties has been evaluated by others (28–31). These histopathological studies have reported that breast tissues from women in their 20s already contain various breast lesions, including ductal carcinoma *in situ*, the presumed precursor lesion of ductal carcinomas. Furthermore, evidence suggests that epigenetic alterations of critical growth-regulating genes, such as methylation of the $p16^{INK4A}$ gene promoter, can be detected in morphologically normal breast tissues (31). Our results extend these previous reports and demonstrate, moreover, that factors released by stromal cells, together with the presumed preexisting changes in the human mammary epithelial cells, suffice to create outgrowths that are indistinguishable from invasive human breast carcinomas. Given the wide spectrum of epithelial histologies observed in these reconstructed human mammary glands, we conclude that this experimental system provides a unique way to study many of the steps of human breast cancer pathogenesis *in vivo*. Moreover, the construction of analogous models of other human epithelial tissues may also prove useful for understanding a variety of other human neoplasias.

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