REVIEW: DEVELOPMENT

Stromal Effects on Mammary Gland Development and Breast Cancer

Bryony S. Wiseman and Zena Werb*

Breast cancer manifests itself in the mammary epithelium, yet there is a growing recognition that mammary stromal cells also play an important role in tumorigenesis. During its developmental cycle, the mammary gland displays many of the properties associated with breast cancer, and many of the stromal factors necessary for mammary development also promote or protect against breast cancer. Here we review our present knowledge of the specific factors and cell types that contribute to epithelial-stromal crosstalk during mammary development. To find cures for diseases like breast cancer that rely on epithelial-stromal crosstalk, we must understand how these different cell types communicate with each other.

The mammary gland comprises stromal and epithelial cells that communicate with each other through the extracellular matrix (ECM). Disruption of communication between the epithelium and stroma can both induce and promote breast cancer. Crosstalk between the mammary epithelium and stroma is also crucial for the proper patterning and function of the normal mammary gland. Interestingly, during its developmental cycle the mammary gland displays many properties associated with breast cancer. Moreover, many of the factors implicated in breast cancer are also vital for mammary development. Understanding how these factors function in normal development may help us to better understand how tumors begin and thrive. Here we review our current knowledge of the various ways in which the stroma and the extracellular environment regulate mammary gland development and tumorigenesis.

Parallels Between Mammary Gland Development and Breast Cancer

Most vertebrate organs are patterned during embryogenesis and then maintain their basic structure throughout adult life. Breast tissue is distinct in that it continually changes in structure throughout the lifetime of reproductively active females (Fig. 1). In the mouse, crosstalk between the epithelium and the mesenchyme specifies the mammary bud at mid-gestation (*1*). The specified mammary epithelium later invades from the nipple into a pad of fatty tissue called the mammary fat pad and forms a small, branched ductal network in the proximal corner of the fat pad. After birth, the epithelium grows in concert with the mouse. Around the time of the release of ovarian hormones at puberty (~3 weeks of age), the distal ends of the mammary ducts swell into bulbous structures composed of multiple layers of cuboidal epithelial cells, called terminal end buds (TEBs) (Fig. 2). The TEBs are the invading fronts of the ducts that proliferate, extend into the fat pad, and branch by bifurcation until the ducts reach the limits of the fat pad, whereupon the TEBs regress (*2*).

The final developmental fate of the mammary gland is fulfilled only when pregnancy and lactation occur. Reproductive hormones induce the expansion and terminal differentiation of the mammary epithelium into secretory, milk-producing, lobular alveoli, and the large fat cells dedifferentiate into tiny preadipocytes (*1*). When the pups no longer suckle on the mammary gland, the secretory epithelium of the mammary gland dies by apoptosis, the fat cells redifferentiate, and the gland is remodeled back to a state resembling that of the adult nulliparous mouse. This process is called involution [(*3*) and references therein] (Fig. 1).

Although it is the mammary epithelium that proliferates, invades, and has the most tumorigenic potential, the mammary stroma contributes both instructive and permissive signals. The mammary stroma consists of multiple components: adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells, and ECM, each subject to regulation throughout the developmental cycle.

The developing mammary gland displays many of the properties associated with tumor progression, such as invasion, reinitiation of cell proliferation, resistance to apoptosis, and angiogenesis. For example, the TEB is a rapidly proliferating mass of epithelial cells that invades into stromal tissue, much like a solid tumor. Furthermore, the epithelium must retain the ability to initiate proliferation throughout its lifetime. Mechanisms also exist within the lactating mammary gland to protect it from premature involution, and therefore it has an inherent mechanism to actively resist apoptotic signals. In addition, as the mammary gland undergoes these morphological changes, the blood supply must be adjusted, and thus, like tumors, the mammary gland induces angiogenic remodeling (*4*). The mammary gland retains many of these properties throughout its lifetime. Thus, it is not surprising that many of the factors essential for mammary gland development (*1*) are also associated with cancer, and that many of these are stromal factors.

The ECM and Stromal Factors Regulate Branching and Tumorigenesis

The control of branching morphogenesis remains one of the most challenging questions in developmental biology. The precise signals that specify new branch points and determine spacing of epithelial ducts remain unclear. In the mammary gland, a variety of genes have been implicated in these processes, and many of these genes are expressed in stromal cells. Many of these genes have also been linked to tumorigenesis.

The mammary gland branches by two mechanistically distinct processes: TEB bifurcation and sprouting of side branches from mature ducts (Fig. 2). During TEB bifurcation, the distal epithelial cells (known as cap cells) abut the fat cells through a sparse basement membrane, and stromal matrix is deposited to form a cleft at the site of bifurcation. In contrast, side branches must extend through the layer of myoepithelial cells, degrade the basement membrane that surrounds the mature epithelial ducts, and invade a periductal layer of fibrous stromal tissue that separates the epithelium from the fat cells of the mammary fat pad (Fig. 2).

Interaction between the epithelium and the ECM plays a major role in mammary gland branching morphogenesis. TEB formation and ductal invasion are disrupted upon inhibition or deletion of factors that regulate the ECM. These factors include two types of receptors for ECM: (i) discoidin domain receptor–1, which can serve as a collagen receptor (*5*), and (ii) β1 integrin, which recognizes many ECM proteins (*6*). In addition, the ECM protein laminin-1 (*6*) and several matrix metalloproteinases (MMPs), which cleave ECM and other proteins in the cellular

Department of Anatomy, University of California, 513 Parnassus Avenue, San Francisco, CA 94143, USA.

*To whom correspondence should be addressed. E-mail: zena@itsa.ucsf.edu

microenvironment (*7, 8*), must function properly. Notably, MMP-mediated cleavage of laminin-5 releases bioactive laminin fragments that induce breast epithelial cells to migrate (*9*). This may be an important mechanism for TEB invasion in vivo.

Proper side branching also requires that the ECM and the cellular microenvironment surrounding the ductal epithelium be maintained. Unrestrained side branching often results in tumorigenesis. Indeed, excessive side branching and eventual tumorigenesis occurs when the stromal regulators MMP-3 (*10*) and MMP-14 (*11*) or the secreted growth/differentiation factor Wnt-1 (*12*) are overexpressed in the mouse mammary gland. In contrast, a reduction in side branching occurs in mice deficient in MMP-3 (*8*) and Wnt-4 (*13*). Wnt-1 or MMP-3 expression also converts the fatty stroma of the mammary gland into a more dense and fibrotic stroma (*10, 12*), and human breast hyperplasia, dysplasia, and carcinoma frequently show elevated stromal MMP activity. Wnts are induced by the progesterone receptor (*13*), which regulates the branching of neighboring cells (*14*), but how this paracrine signal works is unknown. Wnts associate with the ECM, and a cell surface heparan sulfate proteoglycan (HSPG), syndecan-1, is necessary for the phenotype of the Wnt-1 transgenic mice (*15*). These observations suggest that Wnts may mediate the paracrine signal from the progesterone receptor through the ECM and this HSPG. Another stromal factor required for side branching is the actin binding/severing protein gelsolin (*16*). Interestingly, a high expression level of gelsolin is a feature of early-stage but aggressive non–small-cell lung carcinomas (*17*). This suggests a role in invasion; however, gelsolin may regulate other functions as well, because it is markedly down-regulated in ~70% of late-stage human breast cancers (*16*).

Among the stromal factors that function to prevent inappropriate side branching is transforming growth factor β (TGFβ), which is also a key player in tumorigenesis (*18*). TGFβ is present in mature periductal ECM in mice and is specifically down-regulated at sites where side branches are being initiated (*2*). Furthermore, ducts branch excessively when TGFβ receptor signaling within the mammary stroma is inhibited by the targeted expression of a dominant-negative TGFβ receptor [reviewed in (*2*)]. Similarly, mouse studies have shown that the deletion of the myoepithelial cell adhesion molecule P-cadherin causes excessive side branching in addition to mammary hyperplasia and dysplasia later in life (*19*).

Candidate Molecular and Cellular Mediators of Epithelial-Stromal Crosstalk

Several factors have been postulated to directly mediate the crosstalk between the stroma and epithelium during mammary gland development. One such factor is patched-1 (Ptc-1), the receptor for the secreted signaling protein hedgehog (Hh). Haploinsufficiency of Ptc-1 (loss of a single copy of the gene) in mice leads to disruptions in mammary gland development (*20*). Specifically, the mammary ducts of mice haploinsufficient for Ptc-1 show hyperplasia and dysplasia, they become occluded with epithelial cells, and they are surrounded by an unusually dense layer of fibroblastic stroma. However, mammary epithelial transplant experiments have shown that the requirement for Ptc-1 is likely to be stromal rather than epithelial. Interestingly, Indian hedgehog, the probable ligand for Ptc-1 in the mammary gland, is expressed exclusively in the epithelium, which suggests that epithelial Hh may mediate crosstalk from the epithelium to the stroma. In support of this idea, the expression pattern for Gli2 (a downstream target of Hh signaling) is exclusively stromal, and Gli2 is stromally required for normal mammary development (*21*). Ptc-1 is thought to be a negative regulator of Hh signaling, and, in contrast to mammary glands from Ptc-1 mutants, those from Gli2-null mice have a thinner layer of periductal stroma containing few fibroblasts (*21*).

Another candidate factor is parathyroid hormone-related protein (PTHrP), which is required for specification of the cell fates of the nipple and ductal mammary epithelium and mesenchyme as well as ductal branching in both the embryonic and pubertal stages of mammary gland development (*22, 23*). Mammary epithelial cells produce PTHrP, whereas stromal cells express its receptor (*24*); thus, PTHrP likely provides a direct epithelium-to-stroma signal.

A third candidate factor is insulin-like growth factor–I (IGF-I), a requisite factor in mammary gland development. IGF-I is induced by and mediates the function of growth hormone (GH) and the GH receptor [reviewed in (*25*)]. GH and GHR are required for mammary ductal development, yet epithelial expression of GHR in the mammary gland is not required, which suggests that GHR functions in the mammary stroma (*26*). This view is further supported by data showing that GH, acting on the GHR in isolated mammary stroma, induces *IGF-I* mRNA (*27*). In contrast to GHR, the IGF-I receptor (IGF-IR) is required in mammary epithelium for proper ductal development (*28*). Thus, it appears that GH activates GHR in the stroma, thereby inducing stromal expression of IGF-I, which then acts on its receptor in the epithelium.

Which stromal cells produce the signals

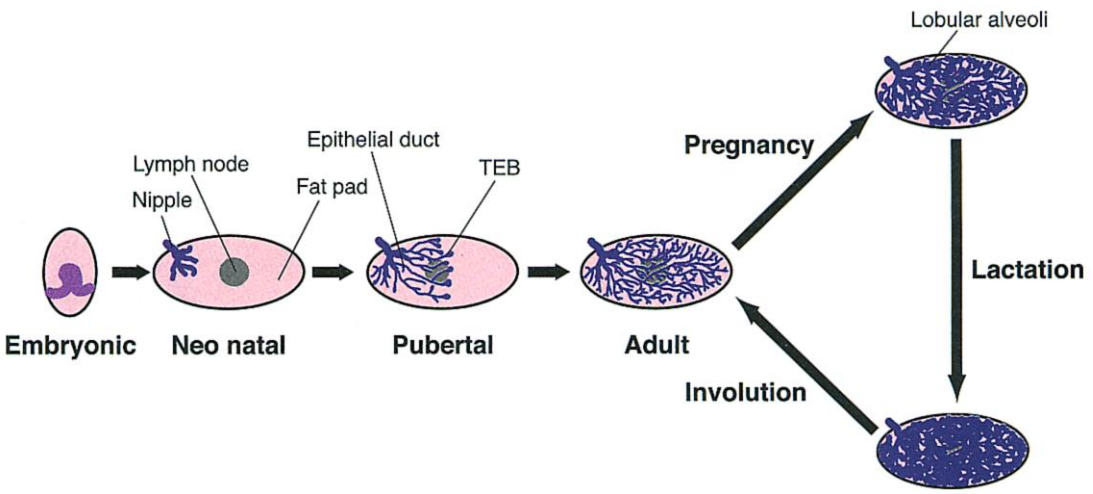

Fig. 1. Stages of mouse mammary gland development. The mouse mammary gland is specified at embryonic day 10. The mammary epithelium invades the fat pad and forms a small, branched ductal network. After birth, the epithelium grows in concert with the mouse but does not begin to fill the fat pad until the release of ovarian hormones at puberty (around 3 weeks of age). With the onset of puberty, TEBs form and the ducts invade, branch, and eventually fill the fat pad by around 10 weeks of age. In the first stage of pregnancy, ducts branch laterally and form side branches with concomitant epithelial proliferation. Alveolar structures then form on the expanded ductal tree and differentiate into lobular alveoli. Finally, the lobular alveoli terminally differentiate and the epithelium becomes secretory, ready to provide milk for suckling pups upon parturition. At this stage, the epithelium has expanded to almost fill the mammary gland and the large fat cells have dedifferentiated into small pre-adipocytes. Upon involution, the secretory epithelium of the mammary gland dies by apoptosis, the fat cells redifferentiate, and the gland is remodeled back to a state resembling that of the adult nulliparous mouse.

that are required for mammary development and tumorigenesis? Inflammatory cells appear to play an important role. Macrophages, recruited by colony stimulating factor–1 (CSF-1), promote mammary ductal invasion during puberty (*29*). CSF-1 is also necessary for the progression of mammary tumors to malignancy in a mouse model (*30*) and during pregnancy for lobuloalveolar differentiation (*31*). It is not yet clear whether macrophage recruitment or CSF-1 signaling is required for malignancy in the tumor model and in lobuloalveolar differentiation. Eosinophils, which are phagocytic cells that are involved in host defenses against parasites and allergic responses, are essential for the proper formation of TEBs (*29*). Conceivably these inflammatory cells could supply bioactive molecules, such as chemoattractants or proteases, or provide specific cellular function, such as phagocytosis.

Metastatic breast cancer cells can target specific organs by mimicking the immune cell targeting mechanism of chemokine attraction. Normally, organs express specific chemokines that correspond to cognate chemokine receptors on the immune cells that need to be recruited. Some human metastatic breast cancer cells overexpress chemokine receptors (CXCR4 and CCR7) and in this way are targeted to organs expressing the paired chemokine ligand (CCL12 and CCL21). These organs—lymph nodes, bone marrow, liver, and lung—are the most common targets for breast metastases. Antibodies that block the interaction of the receptor-chemokine pairs prevent metastasis of these aggressive breast cancer cell lines in mouse models (*32*).

This may not be the only mechanism by which breast cancer cells metastasize to specific organ sites. The most common distal site for breast metastases is bone marrow, and many of the factors that regulate bone development also regulate mammary development. This site may be advantageous for breast cancer cells, given that the stromal environment of the bone marrow shares many characteristics with that of the mammary gland. Bone marrow is a fatty tissue with a rich vasculature, it expresses many of the hormones required for mammary gland development (such as PTHrP and GH), and bone marrow stromal cells express many factors that stimulate survival and growth of stem and progenitor cells of many lineages. The bone marrow would thus provide a familiar stromal niche for breast cells to occupy. The development of the mammary gland and bone are also similar in that breast and bone development are sensitive to the same reproductive hormones and are regulated by similar cytokines. For example, osteoprotegerin ligand (OPGL) and CSF-1 are both needed for differentiation of lobular alveoli and bone-demineralizing osteoclasts (*31, 33*).

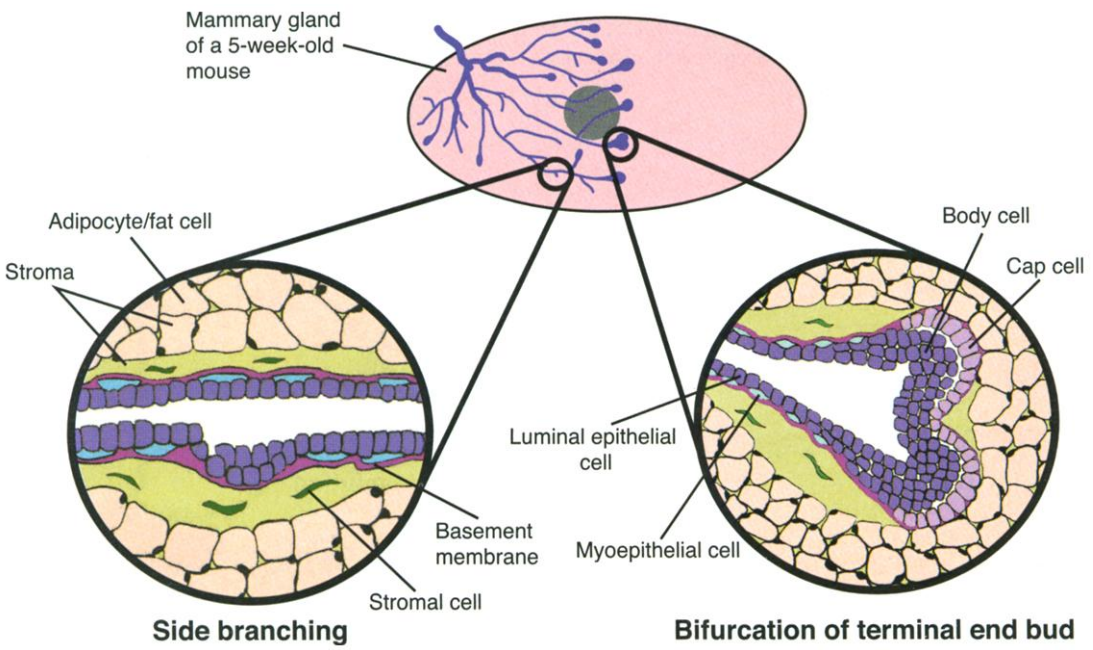

Fig. 2. The two distinct mechanisms of branching morphogenesis in the pubertal mouse mammary gland. The mouse mammary gland branches through two distinct mechanisms: bifurcation of TEBs and side branching. Bifurcation of TEBs to form primary and secondary branches occurs only from immature ducts. The branch point is formed through deposition of stroma at the cleft site, and the ducts extend directly into adipose tissue, without myoepithelial cells or stroma and with only a minimal basement membrane at their invasive front. In contrast, in side branching, a new branch forms from a mature duct. First, the region where the bud is to form must be defined. Then the emerging bud extrudes through and remodels a region containing layers of myoepithelial cells, basement membrane, and periductal stroma. Distinct molecules have been implicated in each type of branching. Factors involved in side branching include the progesterone receptor, Wnts, HSPGs, nuclear factor κB (NFκB), MMPs, TIMP-1, TGFβ and its receptor (TGFβIIR), gelsolin, P-cadherin, CCAAT/enhancer binding protein β (C/EBPβ), CSF-1, Stat5a, and Stat5b. Factors involved in TEB formation include β1 integrin, laminin-1, MMPs, discoidin domain receptor–1 (DDR-1), GH, IGF-I and its receptor IGF-IR, Ptc-1, inhibins and activins, and $p27^{Kip-1}$.

Stromal Regulation of Involution Through Apoptosis and Adipogenesis

The remodeling of the mammary gland after lactation is substantial. During involution, 90% of the epithelium dies by apoptosis and fat cells replace that tissue. There are three stages of involution. In the first stage, individual mammary epithelial cells die by apoptosis, but the general structure of the mammary gland is maintained. This stage is regulated locally by milk stasis, is dependent on the tumor suppressor gene *p53*, and is reversible—that is, suckling can be resumed within 48 hours. The second stage is also characterized by apoptosis, but this is mediated by lactogenic hormones and is independent of *p53*. This stage is irreversible and is dependent on proteinases. The third stage is a biosynthetic phase in which the mammary stroma is remodeled and repopulated with adipogenic cells. The three stages are regulated by distinct mechanisms.

In the first stage, apoptosis is inhibited by the transcription factors Stat5a and interferon regulatory factor–1 (IRF-1) and promoted by the transcription factor Stat3 and the growth factor TGFβ3. Inactivation of either the *Stat5a* or *IRF-1* gene increases mammary gland apoptosis during the first 48 hours after weaning (*34, 35*). In contrast, a reduction in Stat3 protein expression in mice delays apoptosis in the mammary gland (*36*). Stat3 may induce apoptosis by up-regulating a known promoter of apoptosis, IGF binding protein–5 (IGFBP-5), and by down-regulating Stat5a. One proposed inducer of Stat3 is TGFβ3. Mice genetically deficient in TGFβ3 show delayed apoptosis during involution (*37*), whereas mice ectopically expressing TGFβ3 show

premature apoptosis in the lactating mammary gland. This premature apoptosis is concomitant with inappropriate nuclear localization and phosphorylation of Stat3 (*37*). Interestingly, extracellular proteinases regulate the activities of IGFBP-5 and TGFβ3, and thus stromal signals may regulate this stage.

Apoptosis during the second stage of involution likely occurs because the epithelial cells lose their adhesion to a basement membrane, which is destroyed by the increased proteinase activity. As a result, the cells lose survival signals from the ECM. Consistent with this notion, mice that are genetically deficient in plasmin (an extracellular serine proteinase) show reduced apoptosis at 5 days after weaning and exhibit a delay in mammary gland remodeling (*38*). Furthermore, mice that are deficient in tissue inhibitor of metalloproteinases–3 (TIMP-3) show accelerated involution of the mammary gland. In these mice, apoptosis peaks on day 1 of involution rather than day 3, is irreversible, and can be inhibited by an MMP inhibitor (*39*).

Involution is not just about regulation of apoptosis. The mammary gland can also involute more quickly in a situation of reduced proteinase activity, such as in mice that overexpress the metalloproteinase inhibitor TIMP-1 or mice that lack the proteinase MMP-3 (*3*). Here, accelerated involution is due to an increase in the redifferentiation of fat cells rather than an alteration in apoptosis. The regulation of mammary fat cell differentiation is complex. In contrast to MMP-3, plasmin promotes fat cell differentiation. Thus, plasmin-deficient mice have delayed involution as a result of both delayed apoptosis and delayed fat cell differentiation (*38, 40*). Fatty stroma also fosters mammary tumor growth and metastasis (*41*).

Future Prospects

Many aspects of mammary development remain a mystery. Recent work suggests that there is an intimate crosstalk between epithelial development and blood vessel development (*42*). This raises the question of what role the regulation of the vascular supply plays in mammary development and in the development of the adipogenic stroma (*4*). Which molecules are required to recruit stromal cells, and what signals do the stem cells of the mammary gland receive? How do the ducts of the mammary gland signal to each other through the stroma to maintain uniform spacing? How do myoepithelial cells contribute to morphogenesis? What signals regulate the invasion of TEBs, and what signals stop invading ducts and keep them from turning back once they reach the end of the fat pad? What instructs TEBs to regress? How does the crosstalk between the stroma and epithelium evolve during tumorigenesis? Only breast cancer cells can grow in the presence of a normal ductal epithelial network, yet normal epithelial cells can repopulate a gland-free fatty stroma. Which molecules mediate this growth control in normal mammary epithelium or override it in tumors? Obviously, our answers to these questions and others reach beyond just breast cancer research. They will affect our understanding of other types of cancer and many other diseases that rely on a stromal compartment. If our aim is to find cures for diseases that rely on epithelial and stromal crosstalk, then we must increase our understanding of how these different cell types communicate with each other.

References and Notes

1. L. Hennighausen, G. W. Robinson, *Dev. Cell* **1**, 467 (2001).
2. G. B. Silberstein, *Microsc. Res. Tech.* **52**, 155 (2001).
3. C. M. Alexander, S. Selvarajan, J. Mudgett, Z. Werb, *J. Cell Biol.* **152**, 693 (2001).
4. V. Djonov, A. C. Andres, A. Ziemiecki, *Microsc. Res. Tech.* **52**, 182 (2001).
5. W. F. Vogel, A. Aszodi, F. Alves, T. Pawson, *Mol. Cell. Biol.* **21**, 2906 (2001).
6. T. C. Klinowska *et al.*, *Dev. Biol.* **215**, 13 (1999).
7. J. E. Fata, K. J. Leco, R. A. Moorehead, D. C. Martin, R. Khokha, *Dev. Biol.* **211**, 238 (1999).
8. B. S. Wiseman, Z. Werb, unpublished data.
9. N. Koshikawa, G. Giannelli, V. Cirulli, K. Miyazaki, V. Quaranta, *J. Cell Biol.* **148**, 615 (2000).
10. M. D. Sternlicht *et al.*, *Cell* **98**, 137 (1999).
11. H. Y. Ha *et al.*, *Cancer Res.* **61**, 984 (2001).
12. Y. Li, W. P. Hively, H. E. Varmus, *Oncogene* **19**, 1002 (2000).
13. C. Brisken *et al.*, *Genes Dev.* **14**, 650 (2000).
14. C. Brisken *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5076 (1998).
15. C. M. Alexander *et al.*, *Nature Genet.* **25**, 329 (2000).
16. M. R. Crowley, K. L. Head, D. J. Kwiatkowski, H. L. Asch, B. B. Asch, *Dev. Biol.* **225**, 407 (2000).
17. D. B. Shieh *et al.*, *Cancer* **85**, 47 (1999).
18. R. Derynck, R. J. Akhurst, A. Balmain, *Nature Genet.* **29**, 117 (2001).
19. G. L. Radice *et al.*, *J. Cell Biol.* **139**, 1025 (1997).
20. M. T. Lewis *et al.*, *Development* **126**, 5181 (1999).
21. M. T. Lewis *et al.*, *Dev. Biol.* **238**, 133 (2001).
22. J. J. Wysolmerski *et al.*, *Development* **125**, 1285 (1998).
23. J. Foley *et al.*, *Development* **128**, 513 (2001).
24. M. E. Dunbar *et al.*, *Dev. Biol.* **203**, 75 (1998).
25. D. L. Kleinberg, M. Feldman, W. Ruan, *J. Mammary Gland Biol. Neoplasia* **5**, 7 (2000).
26. M. I. Gallego *et al.*, *Dev. Biol.* **229**, 163 (2001).
27. P. D. Walden, W. Ruan, M. Feldman, D. L. Kleinberg, *Endocrinology* **139**, 659 (1998).
28. S. G. Bonnette, D. L. Hadsell, *Endocrinology* **142**, 4937 (2001).
29. V. Gouon-Evans, M. E. Rothenberg, J. W. Pollard, *Development* **127**, 2269 (2000).
30. E. Y. Lin, A. V. Nguyen, R. G. Russell, J. W. Pollard, *J. Exp. Med.* **193**, 727 (2001).
31. J. W. Pollard, L. Hennighausen, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9312 (1994).
32. A. Muller *et al.*, *Nature* **410**, 50 (2001).
33. J. E. Fata *et al.*, *Cell* **103**, 41 (2000).
34. R. C. Humphreys, L. Hennighausen, *Cell Growth Differ.* **10**, 685 (1999).
35. R. S. Chapman *et al.*, *Oncogene* **19**, 6386 (2000).
36. R. S. Chapman *et al.*, *Genes Dev.* **13**, 2604 (1999).
37. A. V. Nguyen, J. W. Pollard, *Development* **127**, 3107 (2000).
38. L. R. Lund *et al.*, *Development* **127**, 4481 (2000).
39. J. E. Fata *et al.*, *J. Clin. Invest.* **108**, 831 (2001).
40. S. Selvarajan, L. R. Lund, T. Takeuchi, C. S. Craik, Z. Werb, *Nature Cell Biol.* **3**, 267 (2001).
41. B. E. Elliott, S. P. Tam, D. Dexter, Z. Q. Chen, *Int. J. Cancer* **51**, 416 (1992).
42. N. Bahary, L. I. Zon, *Science* **294**, 530 (2001).

43. Supported by National Cancer Institute grant CA57621, U.S. Department of Defense Breast Cancer program grant DAMD17-99-1-9113, and Human Frontier Science Program grant RG0051/1999-M. We thank J. Lilla and B. Welm for advice. We apologize to authors whose work could not be cited because of length restrictions.