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Non-autonomous cell proliferation in the mammary gland and cancer

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Abstract

Cells decide whether to grow and divide by integrating internal and external signals. Non-autonomous cell growth and proliferation occurs when microenvironmental signals from neighboring cells, both physical and secreted, license this decision. Understanding these processes is vital to developing an accurate framework for cell–cell interactions and cellular decision-making, and is useful for advancing new therapeutic strategies to prevent dysregulated growth. Here, we review some recent examples of non-autonomous cell growth in the mammary gland and tumor cell proliferation.

Introduction

Classic studies of cell growth and proliferation have used relatively homogeneous populations of immortalized cells due to their experimental tractability. In these cases, cells seem to autonomously decide whether to grow and divide, as microenvironmental cues

are dominated by media formulation and physical cues such as local cells density. These homogeneous cell cultures allow for the detailed analysis of pathways controlling the cell cycle, for example, through gain and loss of function perturbations targeted to specific gene products. However, in multicellular organisms, interactions between heterogeneous cell populations are often the primary regulators of growth during development, normal tissue function, and even in diseases such as cancer. This realization has motivated the development of organoid, co-culture, and tumor xenograft models that provide a means to study so-called non-autonomous cell growth. While all cellular decisions in metazoan are to one degree or another regulated by signals generated by neighboring cells and tissues, we define non-cell autonomous behaviors as those cases where a signal or perturbation impinging on a cell triggers a response in a neighboring cell that may not have received the signal or that may be incapable of receiving the signal. In the case of proliferative control, this means that a signal impinging on one cell type triggers growth and proliferation in a neighboring or secondary cell type. Understanding the principles and mechanisms that regulate non-autonomous cell growth will be critical for gaining deeper insight into the consequences of tumor heterogeneity, epithelial-stromal interactions, and will provide fertile ground for the discovery of new therapeutic targets and strategies. Here, we provide a summary of recent findings related to the non-autonomous regulation of cell growth and proliferation in the mammary gland, and discuss the implications of these findings for diseases such as cancer.

Non-autonomous cell growth in the mammary gland

The mammary gland is a bilayered, tubular epithelial tree. Lining the lumen are milk producing, luminal epithelial cells (LEPs) surrounded by a layer of contractile myoepithelial cells (MEPs). The MEP layer is surrounded by a basement membrane, separating the epithelium from a stroma containing numerous additional cell types such as fibroblasts, adipocytes, lymphocytes, neurons, and endothelial cells [1,2]. Within the luminal layer of the epithelium, there exists a minor population of post-mitotic, hormone sensing cells which range from 7 to 30% in the human mammary gland depending on a host of factors including age, pregnancy history, and exposure to hormones [3]. These cells are generally defined using immunohistochemical (IHC) detection of nuclear localized estrogen receptor alpha (ER α) and progesterone receptor (PR), and are frequently referred to as ER(+) cells [4]. Without dividing themselves [3,5], this minor population integrates hormonal signals produced by the ovaries and coordinates post-natal development and post-pubertal cyclical expansion/regression of the mammary gland via an intricate paracrine signaling network (Figure 1a).

At three different developmental stages – prepubertal, postpubertal, and pregnant – a distinct spectrum of hormones signal through ER(+) cells which in turn send out new signals to the microenvironment that serve to coordinate growth and morphogenesis. At the pre-pubertal stage, for example, estrogen-dependent signaling activates the sheddase activity of the ADAM17 protease to release transforming growth factor alpha (TGF- α) and amphiregulin, Epidermal Growth Factor Receptor (EGFR) ligands, to fibroblasts in the stromal compartment which then signal back to the epithelial compartment promoting ductal tree elongation [2,6,7*]. The complete mechanism of stromal signaling back to the epithelial

compartment remains unclear, but depends on fibroblast growth factors 10 in the stroma and fibroblast growth factor receptors 1 and 2 in the epithelium [8,9]. At the post-pubertal stage, ER α activation is also permissive of PR expression, which when activated, results in local proliferation, primarily via secretion of paracrine signaling molecules: RANKL and Wnt4 [10-12]. Rajaram *et al.* used serial engraftment of mutant mammary epithelial tissue with deletions in either RANKL or Wnt4 in contralateral mouse fat pads and observed that loss of RANKL modestly reduced proliferation, primarily by a reduction in the amount of side branching. By the third serial transplant, loss of Wnt4 secretion led to a drastic decrease in proliferation, more so even than PR deletion. They went on to parse Wnt4 secretion in the perinatal as well as pubertal and adult mammary gland. Their work and others supports a model in which canonical Wnt4 signaling downstream of PR is important for cell self-renewal and drives proliferation of CD44^{high} CK5⁺ luminal progenitor cells required for cyclical expansion and regression in the pubertal and adult mammary gland [11,13]. Though these experiments were conducted in mice, additional work has suggested PR is a key signaling pathway in the human mammary gland as well [10]. Finally, at the pregnant stage, prolactin receptor activation can trigger secondary effects in the epithelium beyond its typical role in stimulating the production of milk proteins such as Beta casein during pregnancy. Recent work by Tarulli *et al.* has demonstrated a unique response of hormone sensing/ER(+) cells to low levels of prolactin due to differences in Wip1 expression, a phosphatase that regulates Jak-Stat signaling. This difference results in hormone sensing/ER(+) cells converting prolactin signals into paracrine growth factors such as RANKL and IGF2 as opposed to milk production [14].

In these three different developmental stages, a rare population of hormone sensing cells within the luminal compartment integrate systemic signals impinging on the gland through the blood stream to drive the exclusive proliferation of their neighbors. In cancer, these and other signaling pathways are frequently co-opted by the tumor and used in a dysregulated manner to promote tumor progression [15]. In invasive breast cancer, for example, ER α signaling is active and necessary for the growth and survival of nearly two thirds of tumors. This observation has driven the development and frequent clinical use of endocrine therapies that block estrogenic signaling, either through competitive inhibitors of ER such as tamoxifen, or through blocking the local conversion of androgens to estrogens via aromatase inhibitors [16]. Strikingly, patients with tumors containing as few as 1% ER+ cells, as defined by IHC staining, still benefit from anti-endocrine therapy [17]. It is interesting that these therapies are effective while presumably targeting such a small proportion of tumor cells, and highlights how non-autonomous proliferation may act as a hub for proliferative control in both the normal and diseased mammary gland. While we still know relatively few mechanistic details governing the estrogenic paracrine signaling circuit in ER (+) breast cancers, particularly a detailed understanding of stromal–epithelial cross-talk, it is likely to be important given its role in normal gland function. It is possible, for example, that estrogenic signaling, in addition to allowing PR expression, is permissive of stromal alterations, creating a permissive microenvironment for tumor growth through mechanisms that remains to be determined. Along these lines, there is evidence that dysregulated RANKL is predictive of disease [18] and there is growing interest in targeting paracrine signaling such as by RANKL inhibition for breast cancer treatment [19,20] though specific

mechanisms remain to be elucidated. Wnt4 has also been implicated as an important factor in cell line models of breast cancer, including in an endocrine resistant model, again paralleling its important role in normal physiology [21,22]. Further insight into these pathways, and the changes they promote within the stromal and epithelial compartments, will likely be fertile ground for novel therapeutic interventions [2,23].

While hormone-sensing cells and their canonical ligands are central to paracrine signaling in the mammary gland, and also play a role in estrogen-dependent breast cancers, recent studies have unveiled additional examples of divisions of labor within the mammary gland that regulate its growth and remodeling. Sonic Hedgehog pathway is a key pathway for epithelial-to-mesenchymal transition across several tissues [24]. Visbal *et al.* demonstrated that overexpression of smoothened, a key transducer of hedgehog signaling, in luminal cells, stimulates proliferation in neighboring luminal cells and also serves to alter the stromal microenvironment, attracting macrophages [25]. This is a phenotype frequently observed in ductal carcinoma *in-situ* lesions (DCIS) and is conceptually analogous to the recruitment of tumor-associated macrophages and promotion of a tumorigenic microenvironment discussed below. A follow-up study by O'Toole *et al.* demonstrated negative survival in a cohort of 279 patients with invasive ductal carcinoma that correlated with the level of hedgehog signaling in the epithelium. They also showed that receptors in the stromal compartment, rather than epithelium, mediated this effect [26].

Mutualism of heterogeneous tumor subclones

With the aid of next generation sequencing technologies, there is now a general appreciation that tumors comprise a heterogeneous mixture of genetically and epigenetically distinct subclones. Tumor heterogeneity can be a consequence of genomic instability and Darwinian competition, but can also arise phenotypically through dysregulated lineage hierarchies [27,28], local variations in microenvironment, and signaling feedback loops [29,30,31*]. There is particular interest in examining intra-tumoral heterogeneity and how different subclones interact via competition or cooperation in order to accurately stratify risk [32], assess metastatic potential [33], and design more precise interventions [34-37]. For example, Marusyk *et al.* developed a panel of 18 MDA-MB-468 subclones, each transduced to overexpress a single secreted factor known to be important for breast cancer progression and transplanted different combinations to investigate clone dynamics. They demonstrated that instead of competing to take over the tumor, a subset of subclones within a polyclonal tumor can collaborate and drive non-autonomous growth or invasion of other subclones (Fig. 1b) [38**]. This concept has been observed in other recent studies as well. Working *in vitro*, Todhunter *et al.* observed that organoids containing a minor subpopulation of RAS-transformed MCF10AT cells enhanced the growth rate of the majority, non-transformed MCF10A cells [39]. In a related experiment with modified MCF10A cells, xenografts containing mixtures of two different lines, one mildly invasive (AT1) and one very invasive (CA1d), produced larger tumors in mouse transplantation studies than a pure population of CA1d or AT1 cells alone. One potential mechanism behind this observation was that the slower growing AT1 line was a source of TGF α and TGF β , driving expansion of CA1ds [40]. Cross-talk between heterogeneous cancer cells can operate through a number of different pathways. Zhang *et al.*, for example, analyzed a (Lin⁻CD29^{High}C-D24^{Low})

subclone population within a murine model of breast cancer and observed higher transcripts of secreted factors Wnt2, Wnt9a, Cxcl12, and IL-6. While these particular cells are not very proliferative themselves, the study demonstrated that blocking these secreted ligands suppressed tumor formation and frequency by inhibiting growth of neighboring cells [41]. These studies add to emerging models of the tumor as organ-like structure which, like normal tissues, have cells serving specialized roles in maintaining tumor expansion, invasion, and survival [42]. Moreover, this more granular view of the tumor will inform new strategies to block growth of heterogeneous tumors.

Cancer associated fibroblasts (CAFs) and tumor associated macrophages (TAMs)

Another mechanism of non-autonomous cell growth is epithelial-stromal cross talk. As described above using estrogen-dependent signaling in the mammary gland as an example, estrogen triggered release of amphiregulin and TGF- α to the stromal compartment is critical for regulating epithelial growth. However, this cross-talk is also a well-documented phenomenon observed during tumor progression and involves tumorigenic cells co-opting normal wound healing processes to promote a proliferative microenvironment [15,43]. This is usually achieved via the secretion of cytokines to recruit blood vessels, fibroblasts, and immune cells. Broadly, once these cells are recruited and activated, they collaborate with tumor cells to support proliferation by two general mechanisms: indirectly by promoting angiogenesis, and more directly by secreting paracrine factors and remodeling the extracellular matrix (ECM).

Tumor associated macrophages (TAMs) are recruited as inflammatory, circulating monocytes by cytokines secreted by tumor cells including CCL2 and CSF-1. Once in the tumor, they differentiate into a secretory, M2-like state [44,45]. The number of local TAMs has been shown to correlate with the proliferation index of the tumor cells as determined by Ki67 staining [46]. Recent work has uncovered numerous cytokines secreted by the TAMs that function to not only drive proliferation in nearby epithelial cells such as IL-6, CCL20, CXCL-1, EGF and IL-10 [47-50], but also extravasation and metastasis [51]. Cancer associated fibroblasts (CAFs) are similarly recruited and activated by secretions from tumor cells such as TGF β and PDGF. They in turn secrete growth factors such as HGF, EGF, and IGF as signaling molecules which serve to drive cell division of neighboring tumor cells [52-54].

In addition to paracrine signaling, another important effect of both TAMs and CAFs is on remodeling the physical properties of the tumor microenvironment by deposition of ECM proteins. CAFs have been shown to enhance the stiffness of the microenvironment and changes to tissue mechanics have separately been shown to increase cell proliferation and invasion Fig. 1c [55]. Alternatively, mutations in macrophages themselves may be sufficient to drive aberrant microenvironmental changes and initiate tumors in other cells. Experiments by Lujambio *et al.* used a mouse model of a conditional p53 deletion in hepatic stellate cells, which serves to remove cell cycle inhibition, and after inducing injury in the liver, demonstrated that the enhanced production of fibrotic tissue resulted in a higher incidence

of tumors but without a change in grade or size [56]. Work by Afik *et al.* using LC/MS-MS suggests that like CAFs, TAMs induce proliferation of epithelial cells by remodeling the microenvironment, specifically by enhancing the deposition of collagen I and XIV [57].

TAMs and CAFs may also modulate the susceptibility of tumor cells to chemotherapeutic drugs and potentially even ionizing radiation by non-autonomous mechanisms, tilting the balance of apoptosis and proliferation back toward proliferation [58-61]. Therefore, interest is growing in targeting these microenvironmental changes directly [62-64] to fight cancer progression and resistance mechanisms. This was explored in seminal work by Straussman *et al.* showing that mutated BRAF melanoma cells are resistant to RAF inhibition when co-cultured with stromal cells that specifically secrete HGF and that *in-vivo*, stromal HGF correlates with poor prognosis [65]. These studies were extended to show that combination therapies including of HGF and RAF inhibition lead to a lower incidence of resistance by removing a key mechanism for rewiring pro-growth signals during drug treatment. In other recent study, combination therapies incorporating fibrosis inhibitors to prevent microenvironmental remodeling by CAFs along with more standard chemotherapeutics was superior at inhibiting tumor growth [66,67]. It now seems clear that factors secreted by the tumor or stroma have a widespread potential to overcome sensitivity to tumor targeted kinase inhibitors, perhaps necessitating the use of such combinatorial treatment strategies [61].

Conclusion and future work

Examples of non-autonomous cell growth, such as those described here in the mammary gland are common in normal physiology. Moreover, there is an increasing appreciation that similar mechanisms likely play an important role in the progression and therapeutic response of cancers. Generally, these pro-proliferative signals take the form of paracrine growth signals or direct modulators of the physical and chemical microenvironment, and recent studies suggest that these pathways can be manipulated pharmacologically for therapeutic benefit. Therefore, a mechanistic examination of non-autonomous cell proliferation is vital in order to enhance our understanding of the complex cellular interactions that regulate normal physiology, as well as to develop the next generation of novel, rationally designed therapeutics.

One important challenge which is beginning to be addressed is developing a better understanding of the difference between stable cell ‘types’ and dynamical cell ‘states’ [68]. Measurements at a fixed time point or tissue state may not be sufficient reveal the dynamical cell-cell interaction landscape, leading to context-dependent, rather than generalizable, findings. Another challenge is identifying the correct *in vitro* or *in vivo* model to examine non-autonomous growth control. Given the complexity of cellular interactions regulating normal and diseased tissues, along with the important differences between human and mouse physiology [69], this can be a particularly vexing problem. However, recent advances in cell culture systems – be they 2D co-cultures, 3D systems with extra cellular matrix mimetics [70], or even ‘organ on a chip’ technologies [71] – are poised to advance our understanding of complex multicellular interactions among human cells. Still, there are significant obstacles to fully deciphering the signaling pathways, both autocrine and

paracrine, in these reductionist models. For example, recent work demonstrated that omitting even a single cell type from an interacting network of cells significantly changed cytokine signaling to make intestinal epithelial cells hypersensitive to TNF α [72]. Other work has demonstrated that vital signaling partners *in-vitro* [73] can be redundant *in-vivo* [74], arguing in favor of work in tractable animal model systems. However, given the differences between human and animal physiology in a number of tissues, translational studies may require new models [69]. Progress in patient derived xenografts (PDX) transplanted into immunocompromised mouse mammary fatpads offers an exciting intermediate model [75]. However, even these can omit key interactions, for example, through the differences between mouse and human stroma [69]. Thus, opportunities exist for advances in the ‘humanization’ of the murine fat pad and other tissues as well. A challenge to the field will therefore be to mitigate the various short-comings of each model by potentially utilizing multiple systems to validate the cellular interactions most relevant to human health and disease.

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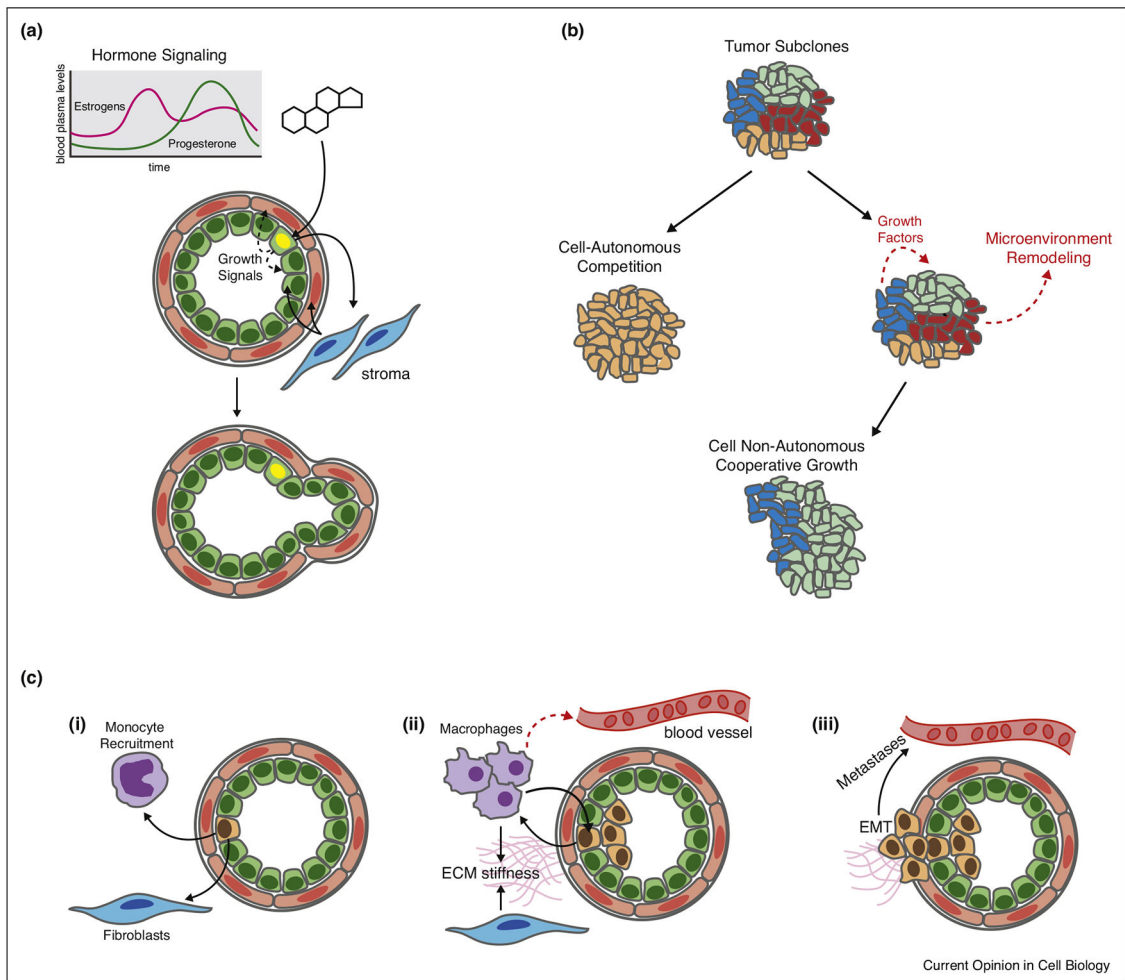
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**Figure 1.**

Examples of non-cell autonomous growth. **(a)** Hormone signaling in the mammary gland. Hormone-sensing cells are restricted to the luminal epithelial lineage (green) and integrate cyclic hormonal signals, such as progesterone. These signals drive proliferation in neighboring cells via paracrine signaling both within the epithelium and through stromal–epithelial cross-talk. This key regulator of mammary gland growth is preserved but dysregulated in many breast cancers. **(b)** Tumor subclones can overgrow/compete (subclone sweep) with the neighbors or cooperate through secreted factors and physical remodeling of the microenvironment. **(c)** Monocytes and fibroblasts can be recruited to tumors by secretion of cytokines, where they mature into secretory macrophages and cancer-associated fibroblasts, which in turn can promote tumor proliferation through the secretion of additional cytokines and other forms of microenvironmental remodeling. This cross talk can enhance invasion, EMT, and ultimately, metastasis (Fig. 1a).