Microenvironmental Regulators of Tissue Structure and Function Also Regulate Tumor Induction and Progression: The Role of Extracellular Matrix and Its Degrading Enzymes

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It is now widely accepted that elements of the cellular and tissue microenvironment are crucial regulators of cell behavior in culture and homeostasis in vivo, and that many of the same factors influence the course of tumor progression. Less well established is the extent to which extracellular factors actually *cause* cancer, and the circumstances under which this may occur. Using physiologically relevant three-dimensional culture assays and transgenic animals, we have explored how the environmental and architectural context of cells, tissues, and organs controls mammary-specific gene expression, growth regulation, apoptosis, and drug resistance and have found that loss of tissue structure is a prerequisite for cancer progression. Here we summarize this evidence and highlight two of our recent studies. Using mouse mammary epithelial cells, we show that exposure to matrix metalloproteinase-3 (MMP-3) stimulates production of reactive oxygen species (ROS) that destabilize the genome and induce epithelial-mesenchymal transition, causing malignant transformation. Using a human breast cancer progression series, we find that ADAM-dependent growth factor shedding plays a crucial role in acquisition of the malignant phenotype. These findings illustrate how normal tissue structure controls the response to extracellular signals so as to preserve tissue specificity and growth status.

INTRODUCTION AND HISTORICAL BACKGROUND

Key questions in cell and tumor biology include how tissues and organs maintain homeostasis, and how cells within organs lose or overcome these controls in cancer. Developmental pathways may be thought of as a series of extremely rapid short-term events in which each new step depends on the previous state with the final outcome at birth being the organism itself. In mice and humans, these developmental events occur in a mere 21 days or 9 months, respectively, but the stability of the differentiated state and the homeostasis of the organism will last 40–110 times longer. Defining how this extraordinary balance is maintained is key to understanding how the stability is lost in the development of malignancy and aging. The importance of “tissue interaction” to define cellular function was recognized as early as the turn of the previous century (Peebles 1911). However, despite the fact that Alexis Carrel published his landmark articles on maintaining cells and tissues outside the animal around this time (Carrel 1908, 1911), intensive investigations of the factors controlling the stability of the differentiated state did not begin in earnest until the early 1970s, with the recognition that the microenvironment has a profound effect on how cells behave in culture (for a review of the early literature, see Bissell 1981). These early studies concentrated on a few molecules gained or lost in culture—the process we loosely define as differentiation.

The role played by the microenvironment in providing a “congenial soil” for tumor growth was identified at the end of the 19th century (Paget 1889), but the discovery of tumor viruses, oncogenes, and tumor suppressors in the 20th century overshadowed these earlier studies as most workers pursued cell-autonomous mechanisms for cancer cell initiation, progression, and metastasis. However, much important work was done in developmental biology, where it was recognized that the epithelial and stromal tissue components engage in a dynamic cross talk during development and play a crucial role in tissue morphogenesis and differentiation. The groundbreaking transplantation experiments in salamanders of Spemann and Mangold demonstrated the power of certain regions of tissues to interact with, and modulate, the differentiation of adjacent structures (Spemann 1918; Spemann and Mangold 1924). Similarly, mammalian tissue coculture experiments defined some of the reciprocal interactions between epithelial and mesenchymal tissues necessary for the induction of the differentiated state in several organs (Grobstein 1964 and references therein; Sakakura et al. 1976). The identities and roles of many of the key signaling molecules have now been elucidated in several organ systems, including the lung (Cardoso 2001), kidney (Yu et al. 2004), gut (Roberts 2000), and mammary gland (Parmar and Cunha 2004).

With the new molecular and genetic tools available to us at the turn of this new century, we are beginning to understand more fully the complexity of the programs and forces that maintain the unity of form and function in normal organs, and the validity of the instability/plasticity of the differentiated state (Bissell 1981; Blau and Baltimore 1991). These tools and techniques have enabled us to

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show that frank cancer cells can be “reverted” to a phenotypically normal state, and how normal tissues may develop epithelial tumors by alterations in the microenvironment and the stroma.

THE IMPORTANCE OF CELLULAR AND TISSUE CONTEXT

That changes in the stromal component play a role in tumor development has long been obvious to surgeons and pathologists. Resection and analysis of tumors show that they are often tough and fibrotic as a result of deposition of collagens and other extracellular matrix (ECM) molecules by stroma. Dvorak noted extensive parallels between tumor-associated stroma and the stroma induced by wound healing, leading him to suggest that “A tumor is a wound that does not heal” (Dvorak 1986). This is referred to as “reactive” stroma, and the mechanisms by which malignant cells induce these changes in nonmalignant fibroblasts are now beginning to be understood (Ronnov-Jessen et al. 1996). The advent of effective means to separate epithelial and stromal cells and recombine them in culture or *in vivo* provided a tool to dissect the contributions of these lineages to the malignant state. It is becoming apparent that the tumor stroma is not merely responsive to the developing tumor, but in fact is an active participant in the tumorigenic process. As with normal development, the interaction between epithelium and stroma in tumor initiation and progression is not a monolog but a dialog, or a highly organized group discussion!

Evidence for a cancer-relevant dialog between epithelium and stroma began to accumulate in the 1970s. DeCasse and coworkers showed that a mammary tumor could be induced to differentiate by coculture (across a filter) with embryonic mammary mesenchyme (DeCasse et al. 1973). A comprehensive series of xenograft experiments by Leland Chung demonstrated that co-injection of either nontransformed or transformed fibroblasts greatly enhanced the tumorigenicity in athymic mice of a series of human cancer cell lines from different tissues (Camps et al. 1990). Similarly, Cunha and his collaborators showed that fibroblasts isolated from the reactive tumor stroma had the capacity to transform otherwise nonmalignant immortal prostatic epithelial cells in this assay (Olumi et al. 1999; Cunha et al. 2004). Conversely, prostate cancer cells could be induced to differentiate when co-engrafted with several different types of mesenchyme (Hayashi et al. 1990).

Although these experiments clearly demonstrated that dialog between epithelium and stroma plays a critical role in neoplasia, the language of communication was still a mystery, including the identity of the paracrine signaling molecules and the cell types which produce these signals. Expression analysis of the different cell populations that comprise the normal and malignant breast is now providing insight into the cells that generate and respond to these signals (Allinen et al. 2004), and it is clear that leukocytes, particularly macrophages, play a prominent role (Bingle et al. 2002). Tumor-infiltrating leukocytes

have long been associated with cancer (Virchow 1863) and were long thought to be a hallmark of the immune response to the tumor. It is now appreciated that these tumor-associated macrophages (TAMs) are actually recruited to the tumor as a result of tumor cell expression of chemotactic cytokines. Once recruited, TAMs provide a rich supply of cytokines, proteases, and growth factors that stimulate tumor growth and promote neo-angiogenesis (Pollard 2004). Yet more signaling proteins, such as MSF produced by fibroblasts in the tumor stroma (Schor et al. 2003), exert potent stimulatory effects on malignant cells.

ORGAN ARCHITECTURE AND CONTEXT AS OVERRIDING TUMOR SUPPRESSORS

The maintenance of tissue architecture and organization, as well as tissue polarity and differentiation, is dependent on continued dialog between the cells and their microenvironment. Loss of organization is among the earliest histological hallmarks of malignant progression. Although the role of activated oncogenes and inactivated tumor suppressors in cancer development is clear, studies of large autopsy series reveal that the majority of people who die from causes other than cancer have small neoplastic growths throughout their bodies (Harach et al. 1985; Nielsen et al. 1987; Montie et al. 1989). It is thus also clear that loss of single tumor suppressors, although necessary, is not sufficient to induce tumors, since inherited mutations such as BRCA1, BRCA2, and APC lead to organ-specific tumors, rather than a general increase in all cancers. What is it that constrains these neoplastic growths and keeps the other tissues intact in the absence of the suppressor genes? Early evidence from a number of laboratories, including ours, has indicated that the tissue microenvironment including its “organ-specific form” can exert a potent tumor suppressive effect, even when cells were infected with potent oncogenes.

In a remarkable experiment performed three decades ago, Beatrice Mintz demonstrated that embryonal carcinoma cells (which form malignant tumors upon injection in adult mice), could contribute to cancer-free normal tissues when injected into blastocysts (Mintz and Illmensee 1975). These data are a striking exposition of the power of the tissue context to modify the malignant potential of cancer cells. Similar and complementary experiments were recently reported by Chin and Jaenisch and coworkers (Hochedlinger et al. 2004). In these, nuclei from ras-transformed melanoma cells were shown to contribute to adult tissues in chimeric mice, although in this case the mice did develop tumors eventually.

Our laboratory focused on Rous sarcoma virus (RSV). This virus, which encodes the potent transforming oncogene, *v-src*, causes sarcoma formation in adult birds and mass transformation of cells in culture (for review, see Martin 2004). We found that infection of embryos *in ovo* with virally encoded Src did not preclude normal embryonic development even though the oncogene was expressed (Dolberg and Bissell 1984), and active (Howlett et al. 1988; Stoker et al. 1990a). Nevertheless, cells iso-

lated from these embryos became transformed en masse when dissociated and placed on tissue culture plastic, demonstrating the importance of context to modify the phenotype of an oncogenically transformed cell. Furthermore, whereas it was widely accepted that viral infection was necessary and sufficient for induction of tumors, we found that RSV-induced tumors would remain local despite circulating virus. We subsequently showed that wounding of infected chickens was in fact necessary and that wounding the chicken at a site different from the original RSV injection was sufficient to induce tumors at the site of wounding. Finally, we showed that TGF- β 1 was a key mediator in this process (Sieweke et al. 1990), one of the early demonstrations of the dual role of TGF- β in normal and malignant behavior. These data demonstrated that oncogene expression was compatible with normal nonneoplastic tissue morphogenesis in the embryo, and that the tumorigenic phenotype could be revealed following microenvironmental perturbations such as those induced by cellular cultivation on tissue culture plastic or wounding in the adult chicken.

Several differences exist in wound healing between the embryo and the adult (Ferguson and O'Kane 2004). Embryonic wounds often heal without scar formation, perhaps because at this developmental stage the skin is actively growing and is rich in morphogenetic growth factors. In adults, the skin is more refractory to healing, which results in scar formation. In contrast to adults, embryonic wounds typically have little inflammatory cell infiltration. The isoforms of TGF- β present at the wound site also differ between embryo and adult. TGF- β 3 is the predominant isoform in embryonic wounds, whereas TGF- β 1 and TGF- β 2 are present in adults. Introduction of TGF- β 3 into adult wounds promotes scar-free healing (Shah et al. 1995), whereas genetic ablation of TGF- β 3 prevents embryonic scar-free healing (Ferguson and O'Kane 2004). These data provide insight into our observations that introduction of RSV by injection in chick embryos did not yield tumors, whereas subsequent wounding of the adult birds, at distant sites, did.

There is much literature (going back to the 19th century) describing the changes that occur in the wounding environment—which included disruption and remodeling of basement membrane (BM), activation of the stroma, immune cell infiltration and degranulation, and, ultimately, scar formation. These events can also provide an oncogenic stimulus to cells with genetic alterations (for a review of the early literature, see Sieweke and Bissell [1994]; for a more recent review see Robinson and Coussens [2005]). But what are the molecular effectors of stability and disruption? One of the problems of trying to define the role of specific components of ECM, BM, or adhesion molecules in development of malignancy is that their very absence is often lethal. Nevertheless, there is increasing evidence that attenuated function of these molecules is indeed instrumental in tumor induction, poor prognosis, and increased progression (Table 1; see the final section of this paper). Dissecting more complex levels of molecular interaction will clearly require robust and more sophisticated models.

THE MAMMARY GLAND AS AN EXPERIMENTAL ORGANISM

To understand the signaling pathways involved in the maintenance of the differentiated state, and to gain insight into the steps that cause and promote malignancy, our laboratory has concentrated for the past 25 years exclusively on one system, that of the mammary gland of mice and humans. We postulated (Bissell et al. 1982), and then demonstrated (for reviews, see Stoker et al. 1990b; Roskelley et al. 1995; Bissell et al. 1999, 2003; Schmeichel and Bissell 2003), that components of the ECM provide critical physical and biochemical cues to epithelial cells, and cooperate with hormones and growth factors to signal to the nucleus and chromatin to maintain mammary-specific gene expression. In parallel, we have been concerned with the loss of tissue organization and polarity, which is among the earliest histological manifestations of an incipient tumor.

The availability of genetic engineering techniques in mice and our ability to culture cells in three-dimensional (3D) gels as a surrogate for normal tissues (Michalopoulos and Pitot 1975; Emerman and Pitelka 1977; Barcellos-Hoff et al. 1989; Petersen et al. 1992) have now made it possible to study the molecular mechanisms involved in how changes in the microenvironment translate into changes in gene expression. One of the first demonstrations that changes in culture substrata and cell shape led to changes in *de novo* gene expression was performed with murine mammary cells and synthesis and secretion of radiolabeled milk proteins (Lee et al. 1984, 1985). Subsequent studies have demonstrated that expression of tissue-specific genes such as milk proteins are not coordinate but that there is a hierarchy of expression directly related to the nature of the ECM molecules, cell–cell interactions, and complexity of the tissue-specific architecture achieved in culture (Fig. 1A) (for review, see Lin and Bissell 1993). Although not all the transcripts have been interrogated in the case of the mouse mammary cells, it is clear that a number of signaling pathways are shut off and others are turned on; i.e., there is change in the program of tissue-specific gene expression when the cells receive the ECM and cell-shape signals. The postulate that these changes are directly related to cell–ECM interactions was demonstrated by studies that showed β -casein expression is disrupted as a function either of inhibition of specific integrins (Streuli et al. 1991; Muschler et al. 1999) or use of specific inhibitory peptides to chains of laminin-1 (Streuli et al. 1995).

Using β -casein as the prototype, we discovered that there is an ECM-response element in the promoter of the β -casein gene (Fig. 1B, C) that responds to laminin-1 and prolactin. The response was quite dramatic and involved recruitment and activation of transcription factors Stat5 and C/EBP β , and widespread alterations in chromatin structure (Schmidhauser et al. 1992; Myers et al. 1998; R. Xu et al., in prep.). We later showed that other genes also contain ECM or substrata-response elements, and in some cases, such as that of the TGF- β 1 gene, the response to ECM was suppression of expression rather than induction (Streuli et al. 1993).

Table 1. Examples of Inherited Genetic Polymorphisms in Microenvironmental Molecules Which Affect Tumor Incidence, Progression, or Survival

Protein class	Gene	Polymorphism	Type	Molecular phenotype	Tumor phenotype	Tissue	Reference
MMPs	MMP1	2G/1G (-1607)	ins/del	2G: high expression	2G: poor prognosis	ovarian	Kanamori et al. (1999)
					2G: poor prognosis	malignant melanoma	Ye et al. (2001)
					2G: poor prognosis	colorectal	Ghilardi et al. (2001)
					2G: more advanced tumors	cervical	Nishioka et al. (2003)
Angiogenesis factors	MMP3	5A/6A (-1612)	ins/del	5A: high expression	5A: increased incidence	breast	Biondi et al. (2000)
	VEGF	G(-1154)A	SNP	G: low expression	AA: reduces susceptibility AA: thinner tumors T/T: increases metastasis	prostate malignant melanoma breast	McCarron et al. (2002) Howell et al. (2002) Ayala et al. (2003)
Adhesion proteins	β3-integrin	T(1565)C	SNP				
	E-cadherin	C(-160)A		A: low expression		bladder stomach	Ayala et al. (2003) Kuraoka et al. (2003)
Cytokines	TNF-α	G(-308)A	SNP		A: more invasive tumors C: more invasive/metastatic AA: worse prognosis	breast non-Hodgkin's lymphoma acute lymphoblastic leukemia	Mestiri et al. (2001) Warzocha et al. (1998); Juszczyński et al. (2002) Lauten et al. (2002)
	TNF-β	A(+252)G	intron SNP		AA: better prognosis AA: better prognosis G: worse prognosis	lung stomach non-Hodgkin's lymphoma	Lauten et al. (2002) Shimura et al. (1995) Warzocha et al. (1998)
	IL-6	G(-174)C	SNP	C: low expression	C: better prognosis C: better prognosis low exp: worse prognosis	ovarian breast	Hefler et al. (2003) DeMichele et al. (2003)
	IL-10	A(-1080)G, T(-819)C, A(-592)C	SNPs	ACC: intermediate expression		cutaneous malignant melanoma	Martinez-Escribano et al. (2002)
				ATA: low expression	low exp: worse prognosis	non-Hodgkin's lymphoma	Cunningham et al. (2003)

Figure 1. Hierarchical control of tissue function by ECM-dependent cell shape changes, signaling, and morphogenesis. (A) Examples of gene products altered by contact with the ECM and by changes in cellular structure. Studies of MECs cultured in the context of a variety of “designer microenvironments” have demonstrated that cells display distinct behaviors in response to changes in cell shape and ECM composition. In these cultures, the inert substratum, polyHEMA, was used to model cell shape change by itself, whereas purified laminin was used as a ligand that stimulates both cell shape change and integrin-dependent signaling. As cells make the transition from 2D to 3D culture, the expression of distinct cassettes of genes is reciprocally modulated. (B) Deletion analysis of the β -casein promoter identifies an ECM-response element (BCE-1). (C) Systematic mutational analysis of BCE-1 demonstrates that the C/EBP β and Stat5 binding sites are required for the ECM-dependent transcriptional response. (Modified, with permission, from Schmidhauser et al. 1992; Roskelley et al. 1995; Myers et al. 1998.)

INTERROGATION OF NORMAL AND MALIGNANT HUMAN BREAST CELLS IN 3D REVEALS THAT SIGNALING REGULATION DIFFERS IN 2D AND 3D

Despite differences in the gross anatomy of mouse and human mammary glands, the assays developed for the mouse cells (Barcellos-Hoff et al. 1989) could be used for human cells with an additional bonus: Normal and non-malignant human breast epithelial cells could be distinguished from premalignant and malignant cells in 3D assays (Petersen et al. 1992). Whereas the normal cells derived from reduction mammoplasty and nonmalignant cell lines became quiescent by day 7 and organized into a replica of human breast acinus with correct tissue polar-

ity and proportions, the malignant cells continued to grow, piled up, and formed large, disorganized, tumor-like colonies (Petersen et al. 1992). Using a unique model of human breast cancer progression referred to as HMT3522 (Briand et al. 1987, 1996), we showed that much of the phenotypic difference between the nonmalignant (S1) and malignant (T4-2) cells was due to changes in the levels and functions of surface receptors for ECM molecules and growth factors leading to aberrant activation of a number of signaling pathways.

Using a series of signal transduction inhibitors and dominant-negative mutants, we have delineated the pathways downstream of receptor tyrosine kinases and adhesion receptors that contribute to the malignant phenotype of T4-2 cells (Fig. 2). Using blocking antibodies against

Figure 2. Reversion of the malignant phenotype by modulation of signaling pathways integrated by the ECM. (A) Schematic representation of the signaling pathways downstream of growth factor and adhesion receptors. The activity of some key proteins (green) is required for the maintenance of the malignant phenotype. (B) The malignant phenotype of T4-2 cells may be reverted in 3D ECM culture using inhibitors of the key signaling receptors and their downstream target proteins described above. A representative example (PI3K inhibition) is shown. $\alpha 6$ -integrin and ZO-1 are markers of basal and apical polarity, respectively. (C) Hierarchical clustering of Affymetrix microarray data of S1 and T4-2 cells, and T4-2 cells treated with inhibitors of the regulatory proteins shown in A. Reversion of the malignant phenotype using diverse inhibitors elicits a common gene expression pattern (P.A. Kenny and M.J. Bissell, unpubl.) (B, Modified, with permission, from Liu et al. 2004.).

β 1-integrin (Fig. 2Ai), we defined a requirement for increased ECM signaling via this receptor for the maintenance of the malignant phenotype (Weaver et al. 1997). Furthermore, we defined a role for increased signaling by the EGFR pathway, since inhibitors of this receptor and downstream signaling molecules effected a similar reversion of the malignant phenotype (Fig. 2Aii) (Wang et al. 1998). This reversion encompassed a restoration of growth arrest in response to ECM, the restoration of apical-basal acinar polarity, the reorganization of the actin cytoskeleton, and the formation of adherens junctions (Fig. 2B). Inhibition of PI3-kinase (PI3K) resulted in a similar phenotype (Fig. 2Aiii), and we have dissected the different contributions of two critical PI3K effectors, Rac1 and Akt, to malignancy in this model (Liu et al. 2004). Because these malignant breast cells lack mutations in common proto-oncogenes (P.A. Kenny and M.J. Bissell, unpubl.), we have probed the extracellular effectors upstream of EGFR to identify the key endogenous growth factors that drive the malignant phenotype. Using this approach we have identified two EGFR ligands and a protease required for their activity, and demonstrated that the malignant phenotype may also be reverted by targeting this protease (Fig. 2Aiv) (P.A. Kenny and M.J. Bissell, in prep.). Importantly, inhibition of any of these pathways down-modulates the others, as well as the level of the surface receptors β 1-integrin, EGFR (Wang et al. 1998), and coxsackievirus and adenovirus receptor, CAR (Anders et al. 2003). This down-modulation does not occur in 2D (Fig. 3) (for review, see Bissell et al. 1999, 2003).

A comparison of cDNA array analysis of nonmalignant and malignant cells indicates that a substantial subset of signaling pathways and programs are altered when cells are placed in 3D (not shown). Furthermore, the array analysis of malignant T4-2 cells treated with these diverse inhibitors indicates that a key subset of genes become "S1-like" in the reverted cells by all the inhibitors tested (Fig. 2C). The contribution of these genes to tissue polarity and growth control is currently under investigation. As such, the reversion model may be used as a screen for how signaling pathways may become integrated as a result of restoration of the tissue architecture. The same approach may be used also for finding new tar-

gets for breast cancer therapy, and a number of laboratories, including ours, are using these assays for such studies at present.

TISSUE POLARITY ALSO REGULATES RESPONSE AND RESISTANCE TO CHEMOTHERAPEUTIC AGENTS

It has long been noted that tumor cells change their response to radiation and chemical treatments when they are aggregated (Kerbel et al. 1996; Durand and Olive 2001). There are well-known pathways by which tumor cells can become resistant to chemotherapy. We reasoned that since tissue structure is so fundamental to phenotypic behavior of tumor cells, one additional mode of resistance could be due to the changes that occur as a result of reestablishment of tissue polarity. That this is indeed the case was shown in a series of experiments we reported in 2002 (Weaver et al. 2002). Briefly, normal and malignant human breast cells become resistant to induction of apoptosis by six different chemotherapeutic agents when they are correctly polarized in 3D (Fig. 4). Both cell types are equally susceptible to cell death when cultivated on tissue culture plastic. That tissue polarity rather than differences in mutation, growth rate, or 3-dimensionality per se is responsible for the observed differences was shown using some of the techniques developed in the laboratory and described above. If normal cells are disorganized in 3D they die. If tumor cells are reverted in laminin-rich ECM, they become resistant; if they are growth-arrested in collagen-I gels where breast tissue polarity is reversed, they still die. These observations were traced to the establishment of the correct tissue polarity through involvement of hemi-desmosomes and α 6/ β 4-integrins (for further details, see Weaver et al. 2002). This is another clear example of the importance of the microenvironment and tissue architecture in function and dysfunction.

	3DlrBM				2D		
	S1	T4-2	T4-2 "treated"	T4-2	T4-2 "treated"	T4-2	T4-2
Inhibitor Added:	-	-	β 1-integrin inhibitor	EGFR Inhibitor	-	β 1-integrin inhibitor	EGFR Inhibitor
β 1-integrin total levels	+	+++	+	+	+++	+++	+++
EGFR total levels	+	++++	+	+	++++	++++	++++
EGFR activated	+	++++	+	+	++++	++++	+

Figure 3. β 1-integrin and EGFR protein levels and activities are coordinately modulated in HMT3522 cells in 3D ECM. This reciprocal cross-modulation is not observed in cells cultured on tissue culture plastic. (Reprinted, with permission, from Bissell et al. 1999 [© AACR].)

Figure 4. Only nonmalignant cells within mammary acini are resistant to apoptosis. Apoptotic labeling indices calculated for S-1 and T4-2 cells treated with Trail peptide (1 μ g/ml), anti-FAS mAb (IgM CH-11, 2 μ g/ml), TNF- α (100 nM), etoposide (50 μ M), cytochalasin B (1 μ M), or paclitaxol (120 nM). Cells were treated (A) as monolayers on a thin coat of collagen I for 24 hr or (B) as 3D structures in rBM for 96 hr. (Reprinted, with permission, from Weaver et al. 2002 [© Elsevier].)

METALLOPROTEINASES AS ONCOGENES; NORMAL STROMA AS A TUMOR SUPPRESSOR

Matrix metalloproteinases (MMPs) regulate a number of pathways relevant to cancer biology, including proliferation, differentiation, apoptosis, migration, and invasion (Egeblad and Werb 2002). Establishment of a causal relationship between MMP overexpression and tumor progression has prompted the development of several MMP inhibitors as anticancer therapeutics, and although clinical trials in which these agents were used to treat late-stage tumors have been disappointing, accumulating evidence suggests that the tumor-promoting activity of MMPs may be most important in the earliest stages of tumor development (Coussens et al. 2002; Egeblad and Werb 2002). We previously observed that transgenic mice expressing an autoactivated form of MMP-3 (stromelysin-1) under control of the whey acidic protein promoter (WAP) to direct expression in mammary epithelial cells in mid-pregnancy (Sympson et al. 1994) develop invasive, genomically unstable mammary tumors (Sternlicht et al. 1999). Development of a culture assay to dissect these effects revealed that treatment with MMP-3 causes mouse mammary epithelial cells to alter cellular morphology, decrease cell-cell interactions, increase motility and invasiveness, down-regulate epithelial cytokeratins, and up-regulate mesenchymal vimentin (Boudreau et al. 1995; Lochter et al. 1997a,b), characteristics of epithelial-to-mesenchymal transition (EMT) that facilitate tumor invasion and metastasis (Thiery 2002). The genomic alterations induced by MMP-3 are strikingly nonrandom, with common patterns of genomic deletions found on chromosome 4 in tumor tissue derived from the WAP-MMP-3 transgenic mice (Fig. 5A) and in cultured mouse mammary epithelial cells exposed to MMP-3 (Fig. 5B) (Sternlicht et al. 1999; Radisky et al. 2005).

A common mechanism underlying both induction of EMT and the development of genomic instability by MMP-3 was suggested by the concomitant appearance of EMT (Fig. 6A) with increased resistance to N-(phosphon-

acetyl)-L-aspartate (PALA) (Fig. 6B) (Johnson et al. 1976), as resistance to PALA is acquired through amplification of the CAD gene (Wahl et al. 1979; Radisky et al. 2005). Our investigations revealed that both the MMP-3-induced phenotypic alterations (e.g., induction of EMT) and genetic changes (e.g., genomic instability) were caused by increases in cellular reactive oxygen species (ROS). Cultured mouse mammary epithelial cells treated with MMP-3 showed significantly increased oxidative DNA damage that was inhibited by treatment with ROS inhibitor N-acetyl cysteine (NAC) and, consistent with the implication that the ROS-mediated DNA damage was related to the induction of genomic instability, we found that NAC inhibited the MMP-3-induced acquisition of PALA resistance, and that elevating cellular redox levels in the absence of MMP-3 by treatment with H₂O₂ was sufficient to stimulate PALA resistance (Radisky et al. 2005). ROS have been implicated as secondary signaling intermediates that control cellular phenotype (Droge 2002; Puri et al. 2002; Finkel 2003), and we found that MMP-3-induced ROS were both necessary and sufficient to induce the EMT phenotypic alterations in target mammary epithelial cells (Radisky et al. 2005).

Previous studies (Kheradmand et al. 1998; Werner and Werb 2002) showed that alterations in cell morphology, a process related to MMP-3-induced EMT, can lead to increased cellular ROS through a Rac1-dependent mechanism. Using pull-down activity assays, we found that MMP-3 treatment stimulated an increase in Rac1 activity and the induction of a higher-molecular-weight form of active Rac1 (Fig. 6C). Rac1b is a highly activated splice isoform of Rac1 that has been found in breast and colorectal tumors (Matos et al. 2003; Fiegen et al. 2004). Quantitative RT-PCR using oligonucleotide primers specific for the Rac1b isoform performed on cells that were treated for up to 4 days with MMP-3 showed a progressively increasing expression of Rac1b for as long as MMP-3 was present, and that Rac1b levels fell off rapidly after MMP-3 withdrawal (Fig. 6D). Consistent with the implication that the MMP-3-induced alterations in Rac1b expression are the trigger for EMT, we observed that expression of Snail and vimentin paralleled the expression of Rac1b (Radisky et al. 2005), and that inhibition of Rac1b expression by RNAi blocked the induction of EMT by MMP-3 (Fig. 6E).

A contrast can be drawn between the phenotypic consequences of MMP-3/Rac1b-induced ROS (i.e., induction of EMT) and the genetic consequences (i.e., DNA damage and genomic instability). Although it seems likely that MMP-3-induced genomic instability is a nonspecific or pathological response, we conjecture that induction of EMT by MMP-3/Rac1b-produced ROS is caused by specific activation of select signaling pathways; indeed, we found that MMP-3-induced ROS were both necessary and sufficient for induction of the Snail transcription factor, implicated previously as a key mediator of EMT (Nieto 2002; Thiery 2002). Although we have shown that MMP-3 can directly cause malignant transformations, it is noteworthy that MMPs are involved in many normal developmental processes, and that genetic deficiency of MMPs

Figure 5. Nonrandom patterns of genomic instability induced by treatment with MMP-3. (A) Analysis of normal and tumor tissue from WAP-MMP-3 transgenic mice by cytogenetic comparative genomic hybridization (CGH) reveals common patterns of deletions (red) in chromosome 4. (B) Cultured SCp2 mouse mammary epithelial cells exposed to MMP-3 become genomically unstable and show deletions in chromosome 4, as revealed by array-based CGH. (A, Adapted from Sternlicht et al. 1999; B, adapted from Radisky et al. 2005.)

Figure 6. Induction of EMT and genomic instability by treatment with MMP-3: Role of Rac1b. (A) MMP-3-treated SCp2 cells, stained for cytokeratins (red), vimentin (green), and DNA (blue); bar, 50 μ m. (B) Induction of PALA resistance by MMP-3 (green diamonds, MMP-3; red squares, untreated). (C) Treatment with MMP-3 stimulates production of Rac1b, a highly activated splice isoform of Rac1. (Top) Active Rac species as assessed by PAK pulldown assay; (middle) total cell lysates probed with antibody raised against Rac1b splice insertion; (bottom) total cell lysates probed with Rac1 antibody, showing low levels of Rac1b expression relative to Rac1 in lysate from treated cells. (D) Time course of Rac1b expression in response to MMP-3 treatment and withdrawal. MMP-3 treatment (days 1–4) and washout (days 5–6) (green diamonds, MMP-3-treated; red squares, untreated). (E) Specific suppression of Rac1b by siRNA blocks MMP3-induced EMT. (Adapted from Radisky et al. 2005.)

can lead to an array of developmental abnormalities (Sternlicht and Werb 2001). In this regard, it is relevant that many other developmental signaling molecules have also been found to facilitate tumor progression, including BMPs, TGF- β (Waite and Eng 2003), Wnts (Moon et al. 2002), Notch (Radtke and Raj 2003), and Hedgehog (Pasca di Magliano and Hebrok 2003). The investigation of the tumor-promoting effects of these factors has been facilitated by an understanding of the cellular signaling mechanisms by which they operate, and our new understanding of the MMP-3-induced signaling pathways provides insight into this parallel process. Since our initial studies on MMP3-induced tumorigenesis, two further extracellular proteases, MT1-MMP and matriptase, have been causally implicated in tumorigenesis using transgenic models (Ha et al. 2001; List et al. 2005).

Demonstrating that these stromal alterations actually facilitate tumor progression is difficult in human studies; however, a number of recently developed animal models address this point and show that genetic lesions in the stroma can make a positive and even causative contribution to epithelial tumorigenesis. Conditional ablation of the *Tgfr2* gene in fibroblasts induced the onset of neoplasia in the prostate and squamous cell carcinoma of the forestomach (Fig. 7) (Bhowmick et al. 2004). It has also been shown that growth of MCF7 xenografts is significantly enhanced in SCID mice null for p53. p53^{+/−} mice exhibit intermediate kinetics of tumor growth and, importantly, loss of the wild-type p53 allele was detected in the tumor stroma arising in six of eight tumors in the heterozygous mice (Kiaris et al. 2005).

Figure 7. Signaling pathways in stromal cells activated by TGF- β and BMP prevent tumor formation in epithelia. TGF- β signaling can be initiated by association with type III TGF- β receptors prior to formation of an active kinase complex containing receptor types I and II. The active TGF- β signaling complex phosphorylates receptor-regulated R-SMADs such as Smad2 and Smad3, which then bind to common partner co-SMADs such as Smad4. The resulting activated complex can bind to specific DNA sequences and influence the transcription of many tissue-specific genes. Similarly, BMP family members form an active BMP signaling complex, which phosphorylates Smads 1, 5, and 8. These activated R-SMADs then associate with Smad4 to form an active transcriptional complex. Loss of TGFRII, Smad4, or BMPRI in stromal cells can stimulate the formation of tumors in adjacent epithelium, although the signals involved in this process have not yet been identified. (Reprinted, with permission, from Radisky and Bissell 2004 [© AAAS].)

Figure 8. Dynamic reciprocity: The whole is greater than the sum of its parts. Gene expression and function of differentiated tissues is an emergent property arising from the bidirectional flow of biochemical and mechanical signals between the ECM and the nucleus, transduced by transmembrane receptors, signaling molecules, and cytoskeletal elements. In the last analysis, however, the organ itself is the unit of function (Bissell and Hall 1987).

That not only stroma, but the different epithelial cell types, and in fact all cell types that make up the architecture of the organs (Fig. 8), play important roles in the integrity of the function of that organ can be demonstrated further with studies on the role of myoepithelial cells in establishment of tissue polarity in breast acini (Gudjonsson et al. 2002; Adriance et al. 2005).

INHERITED POLYMORPHISMS IN GENES ENCODING MICROENVIRONMENTAL PROTEINS THAT MAY ALTER CANCER SUSCEPTIBILITY AND/OR PROGRESSION KINETICS

Loss of function of tumor suppressor genes such as RB, BRCA1, BRCA2, and CHK2 is a critical component of a large proportion of familial cancers. However, it is clear that other low-penetrance alleles also contribute to this polygenic disease in the wider population. Several case-control studies have examined the effects of germ-line polymorphisms in genes encoding proteins present in the tissue microenvironment to determine whether variation in any of these genes contributes to an increased risk of incidence or progression in several cancers. Such polymorphisms may alter the coding sequence of the protein, with resultant effects on its activity, or they may occur in noncoding regions of the gene, where they may lead to al-

tered levels of gene expression and/or alternative splicing by creating or deleting consensus binding sites for nucleic acid interacting proteins. Although there has been an explosion of interest in this field, it is important to note that many of these studies are relatively small and, despite achieving statistical significance at the threshold set by the investigators, remain to be confirmed in larger studies. We believe that it is important to be fully cognizant of the statistical problems inherent in this methodology, particularly of the propensity of these studies to produce false positives (Risch 2000; Pharoah et al. 2004).

For the purposes of this paper, we confine our discussion to those SNPs which have been demonstrated (or at least imputed) to exert an effect on protein expression or activity. Several of these studies have been reviewed in detail elsewhere (Balasubramanian et al. 2002; Loktionov 2004) and are summarized in Table 1. Polymorphic changes have been reported in genes encoding growth factors, adhesion receptors, proteases, and cytokines—proteins that play crucial roles in the intercellular milieu. Each of these proteins has been implicated in neoplasia. Several of these SNPs are found in the promoter regions of their respective genes and have been associated with alterations in the mRNA expression level for that allele. Although it remains to be seen how many of these findings will be reproduced in larger, independent populations, these data support the hypothesis that dose-dependent alterations in components of the tissue microenvironment may play causative roles in tumorigenesis or may facilitate the neoplastic progression of an incipient tumor cell.

In addition, the importance of epithelial-stromal interactions for cancer progression is underlined by the detection of clones of mutant stromal cells, the expansion of which has been favored by the acquired mutation(s). Several global studies using microdissected samples of tumor epithelium and stroma with subsequent analysis of loss of heterozygosity or allelic imbalance have shown that distinct genetic alterations occur in the epithelial and stromal compartments. Somatically acquired genetic alterations in the stroma have been reported in cancers of the breast (Moinfar et al. 2000; Kurose et al. 2001; Ellsworth et al. 2004; Fukino et al. 2004), colon (Wernert et al. 2001), bladder (Paterson et al. 2003), prostate (McCarthy et al. 2004), and ovary (Tuhkanen et al. 2004). These genomic studies provide a bird's-eye view of the nature and scale of the differing genetic lesions present in the epithelial and stromal compartments. A greater insight into the co-selected mechanisms that drive tumor progression will come from a detailed molecular analysis of these regions, and recent studies have shown that some of the players most familiar from epithelial tumorigenesis are also altered in the stroma. Mutations in p53 and PTEN have been reported in the stroma of breast carcinomas (Kurose et al. 2002), and mutations in EGFR also have been detected in this compartment (Weber et al. 2005). Given the number of mutational and cytogenetic abnormalities detected in stroma to date, it seems likely that epigenetic alterations in stromal cells might also contribute to tumorigenesis. Collectively, these striking data suggest that

there is a strong selective pressure in tumors of different epithelial origins for the coevolution of a clonal stromal compartment.

ACKNOWLEDGMENTS

These investigations were supported by grants from the U.S. Department of Energy, Office of Biological and Environmental Research (DE-AC03 SF0098), National Cancer Institute (2 R01 CA064786-09), and an Innovator Award from the Department of Defense Breast Cancer Research Program (BC012005) to M.J.B.; and from the National Cancer Institute (CA57621) to M.J.B. and Zena Werb; and by postdoctoral training fellowships (Susan G. Komen Breast Cancer Foundation #2000-223 and DOD BCRP DAMD17-00-1-0224 to P.A.K. and American Cancer Society PF-02-009-01-DDC to D.C.R.).

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Cold Spring Harb Symp Quant Biol 2005 70: 343-356

Access the most recent version at doi:[10.1101/sqb.2005.70.013](https://doi.org/10.1101/sqb.2005.70.013)

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