MINI REVIEW

TUMOR REVERSION: CORRECTION OF MALIGNANT BEHAVIOR BY MICROENVIRONMENTAL CUES

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Cancer is characterized by unrestrained proliferation and loss of organization, a process that is intimately linked to, and controlled by, genic signaling between the genetically altered tumor epithelium, the stroma, the components of the basement membrane and inflammatory mediators. Much work has been done to characterize the genetics of cancer cells. In this review, we describe the experiments that have been performed, which point to the significant role of the tissue microenvironment in the developmental regulation of normal and neoplastic cells. Using a variety of model systems, the works of a number of laboratories have converged on a hypothesis where the correction of 1 or 2 signaling defects can revert tumor cells to a normal phenotype, both in vivo and in culture, even when the tumor cells possess multiple genetic and epigenetic lesions. This paradigm has been successfully used to treat acute promyelocytic leukemia, and it remains the task of biomedical researchers to identify additional targets for the reversion of other human malignancies.

Key words: breast cancer; 3D models; engineered mice; extracellular matrix; microenvironment

Over several decades, our understanding of the pathogenesis of neoplasia has been advanced tremendously. Many oncogenes and tumor suppressor genes have been identified and characterized, and it is usually accepted that cancer is a genetic disease. Nevertheless, it is beginning to be appreciated that the interrelationships between the tumor epithelium and the tissue microenvironment play a critical role in tumorigenesis. While much work remains to be done, we now have at our disposal many sophisticated tools with which to probe the interactions between the tumor and its host. Here we briefly describe the early seminal studies that demonstrated the ability of the tissue microenvironment to control malignancy and discuss the approaches that have been taken to explore the mechanisms of tumor initiation, progression and regression in physiologically relevant model systems.

The observation by Stevens and Little in 1950s of a high frequency of spontaneous testicular teratocarcinomas in a strain of inbred mice paved the way for critical advances in cancer biology over succeeding decades. In addition to undifferentiated embryonal carcinoma (EC) cells, these tumors consist of nervous tissue, epithelium, cartilage, bone, muscle, fat and glandular tissue. Stevens and Little succeeded in establishing a rapidly growing, transplantable tumor consisting primarily of undifferentiated EC cells. The pluripotency of EC cells was established by Kleinsmith and Pierce, who showed that injection of a single undifferentiated EC cell could give rise to numerous tissue types.

Using coat color as a marker, Brinster demonstrated in 1974 that EC cells (which form malignant tumors upon subcutaneous injection) could contribute to the development of chimeric mice if injected into the blastocyst. These findings were confirmed and extended by Mintz and Illmensee, who used isoenzyme analysis to show that EC cells injected into the blastocyst contributed to the formation of a variety of tissues in cancer-free adult mice. Collectively, these demonstrations that otherwise malignant cells could contribute to normal structures provided a striking exposition of the power of tissue context to modify the malignant potential of cancer cells. These exciting findings were, however, eclipsed by the discovery of the genetic material of the first oncogene, Src, in 1970, the later discovery of cellular protooncogenes, the demonstration that the transformed phenotype could be transmitted by transfer of DNA from transformed cells and the subsequent race to identify and characterize the role of these additional mutant genes. These early studies had been interpreted to suggest that genetic alterations were not necessarily needed for tumorigenesis, a finding hard to reconcile with the new genetic discoveries. Therefore, the implications of these experiments, that genetic alterations could be trumped by the microenvironment, were not widely appreciated as the oncogene paradigm and the importance of genetic changes in cancer rapidly took hold.

During the 1980s, our laboratory focused on understanding the role of tissue context in the malignant transformation of cells by Rous sarcoma virus (RSV). This virus, first characterized in 1911, had been shown to transform cells efficiently and rapidly in culture and to cause sarcomas upon injection in chickens. That there was indeed an oncogene that was responsible for the transformation was proven in the 1970s by Duesberg and Vogt by chemical analysis of DNA from transforming and transformation-defective RSV and by Martin, who isolated a temperature-sensitive mutant of RSV. Later, it was shown that the activity was mediated by the nonreceptor protein tyrosine kinase, pp60-src. Our data, inspired by the earlier studies of Milford and Duran-Reynals, showed that infection of chick embryos in ovo with RSV did not lead to malignant transformation, even though v-Src was both expressed and active. Cells explanted from these embryos rapidly became transformed in culture. In chickens, RSV-induced tumors typically form at the viral infection site. Additional experiments in our laboratory showed that this wounding was required for local transformation, and that additional tumors could be induced at distant sites simply by wounding the infected birds. These experiments indicated that factors involved in wound repair and tissue remodeling had a cocarcinogenic effect in RSV transformation, and that as long as tissue architecture was not disrupted, RSV-infected cells did not become malignant. We subsequently showed that administration of TGF-β, a factor that plays a role in wound healing, was sufficient to induce tumor formation in vivo.


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the RSV-infected chickens, a surprising finding at the time since TGF-β had been shown to inhibit epithelial cell growth potently in vitro, although wounding itself had been implicated as a possible cocarcinogen for decades. Collectively, these experiments with RSV demonstrated that factors specific to the environment of the cell were required to attenuate, or to facilitate, the transforming activity of this potent oncogene. Recent work in transgenic models and specialized cell culture systems has begun to define the specific microenvironmental determinants that have the power to normalize overtly malignant cells.

IN VIVO AND CULTURE MODELS OF REVERSION

Increasingly elaborate and physiologically relevant models are now being employed for functional studies. While each method has distinct advantages and disadvantages, together they provide a powerful complementary approach that has yielded much insight into the processes of tumorigenesis.

For many years, our laboratory has sought to understand the mechanisms by which cells respond to their microenvironment, and how these signals are integrated to specify programs of gene expression and ultimately tissue phenotype. Understanding these processes, and their alterations during neoplasia, will engender a more sophisticated appreciation of the mechanisms involved in tumor progression. We have concentrated on the mammary glands of mice and women as our experimental system and have developed a series of models with which to address these questions.

The normal mammary gland is composed of a double layer of cells: an inner layer of secretory luminal epithelial cells surrounded by a layer of myoepithelial cells. This bilayer is surrounded, in turn, by a basement membrane (BM), which separates the epithelial and stromal compartments. We have previously shown that normal mouse mammary epithelial cells and human primary breast epithelial cells differentiate morphologically and functionally when cultured in a 3D laminin-rich basement membrane (3D lrBM, Matrigel). Matrigel is a solubilized basement membrane gel extracted from the Engelbreth-Holm-Swarm mouse sarcoma and consists primarily of laminin 1, collagen IV, heparan sulfate proteoglycan, nidogen and entactin. When grown in this substratum, normal human primary breast epithelial cells form polarized, growth-arrested multicellular structures with central lumena that are strongly reminiscent of mammary acini in vivo. The cells in these structures express markers of luminal epithelial cells (keratins 18 and 19) and deposit an endogenous basement membrane. In contrast, several primary carcinoma cultures and carcinoma cell lines tested failed to differentiate in this manner and formed continuously proliferating, disorganized structures in 3D lrBM. This assay thus serves to discriminate effectively and rapidly between normal and malignant breast epithelial cells in culture.
The laminin component of lrBM is a critical determinant of morphogenesis and differentiation and is necessary and sufficient to direct tissue-specific gene expression and functional differentiation of mouse mammary epithelial cells in culture. Similarly, laminin 1 specifies the correct formation of polarized acini by human mammary epithelial cells in 3D cultures. Antibody-mediated inhibition of \( \beta\)-integrin, the laminin 1 receptor, blocked acinar morphogenesis of normal human mammary epithelial cells in 3D lrBM cultures. When normal human primary mammary epithelial cells are cultured in a collagen I matrix, they form inversely polarized structures as judged by their altered expression of sialomucin, epithelial-specific antigen and occludin. The polarity of these inside-out structures can be corrected either by the addition of laminin or by coculture with myoepithelial cells. Myoepithelial cells appear to be the only cells in the breast that express laminin 1, and we have proposed that they play a crucial role in the specification of epithelial polarity and suppression of the malignant phenotype in the breast \textit{in vivo}. These 3D assays have been useful for the identification of factors that influence the malignant properties of cells. Our first surprise was with the metastatic cell line, MDA-MB-435, thought at that time to be derived from a breast carcinoma, but now suspected to be derived from a melanoma. Using a candidate gene approach, together with the laboratory of Patricia Steeg, we examined the effects of the expression of a putative metastasis suppressor gene, NM23-H1, in these cells. Expression of this protein allowed the cells to regain many properties observed with normal mammary cells in our 3D lrBM assay. The cells formed growth-arrested, acinus-like spheres in 3D culture and expressed and basally deposited a collagen IV-, laminin-containing basement membrane. Tumors formed by NM23-H1 transfectants upon subcutaneous injection had been shown to produce significantly fewer metastases than the parental cell line.

Our more recent investigations have utilized the HMT-3522 breast tumor progression model. The HMT-3522 cell series originated from a purified epithelial cell population recovered from a breast biopsy of a woman with benign fibrocystic disease and was subjected to sequential passages for over 10 years. Early passages of the cells, termed S1, are nontumorigenic and form phenotypically normal structures in 3D lrBM. Serial passage in the absence of epidermal growth factor led to the outgrowth of a population of cells that formed tumors in athymic mice. After 2 rounds of xenografting, a population was explanted, termed T4-2, that was malignant \textit{in vivo} and formed disorganized continuously growing structures in 3D lrBM. We have established other sublines with phenotypes intermediate between the S1 and T4-2 cells and these represent additional reagents with which to model and understand the behavior of premalignant cells in 3D cultures. The S1-to-T4-2 progression series, derived from the same individual and consequently of the same genetic background, provides an effective model to dissect mammary acinar morphogenesis, polarity and tumor progression and the role of the extracellular matrix (ECM) in these processes. S1 and T4-2 cells have been partially characterized cytogenetically and extensive efforts are underway in our laboratory to identify the gene expression profiles of these cells.

We have used the HMT-3522 cell series to investigate the signaling pathways that are deregulated in malignant cells and have shown that we can revert these cells to a near-normal phenotype with much reduced tumorigenicity using multiple agents. Having observed increased relative amounts of \( \beta\)-integrins on T4-2 cells when compared to the nonmalignant S1 cells, the T4-2 cells were treated with a function-blocking antibody to \( \beta\)-integrin. These cells underwent a striking morphological reversion in 3D lrBM, becoming visually indistinguishable from the acinus-like structures formed by the nonmalignant S1 cells. Attenuation of

**Figure 2** – Contribution of v-Src-infected cells to normal structures during chick embryo development. Chick limb buds were infected at day 4 \textit{in ovo} (embryonic stage 24) with a virus encoding v-Src and a genetic marker, beta-galactosidase. The contribution of v-Src-infected cells to normal tissues (in this case a day 14 feather filament) is revealed by X-gal staining of embryo whole mounts (data not shown; see also Stoker \textit{et al.}.)
attenuation of EGFR signaling, using either a function-blocking antibody (mAb225) or a pharmacological inhibitor (tyrphostin AG1478), led to the phenotypic reversion of the disorganized colonies to growth-arrested polar acinar structures with a concomitant downregulation of β1-integrin levels. Furthermore, the inhibition of MAPK kinase activation (G9080B9), an event downstream of both EGFR and β1-integrin, also resulted in phenotypic reversion. As with the β1-integrin-mediated reversion, this phenotype was reversible when the cells were repropagated as monolayers and subsequently cultured in 3D IRBM in the absence of the inhibitors. It is important to note that this reciprocal cross-modulation of both the levels and activities of EGFR and β1-integrin was not observed in 2D cultures, indicating that the pathways downstream of these factors are integrated only when the cells receive cues from the ECM in a physiologically correct 3-dimensional context.42–44 Such observations demonstrate the importance of using physiologically relevant models for the study of how signaling pathways are integrated in tissues and illustrate the data that may be lost when cells are cultured on 2D plastic substrata.

In addition to integrins, other receptors are required for the cell to interpret signals from the extracellular matrix. One such factor is the basement membrane receptor, dystroglycan, an essential signal for functional differentiation of mammary epithelial cells.45 Work from others and our laboratory has shown that α-dystroglycan (α-DG) is frequently lost in breast carcinomas,46,47 and we showed that restoration of α-DG expression in T4-2 cells is sufficient to allow these cells to form polar, growth-arrested acini in 3D IRBM culture.47 Significantly, overexpression of α-DG blocked the ability of T4-2 cells to form tumors upon subcutaneous injection in nude mice.

In a similar vein, recent work from Kirshner et al.48 has shown that expression of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in the MCF7 human breast carcinoma cell line was sufficient to allow these cells to undergo morphogenesis to form a single layer of cells surrounding a central, apically cleared lumen, although the generation of apicobasal polarity and the malignant status of these structures have not yet been addressed.

We have recently shown that the malignant T4-2 cells overexpress the coxsackievirus and adenovirus receptor, CAR, and that this overexpression correlated strongly with loss of tissue integrity and polarity. The difference in CAR levels between normal and malignant cells was observed only in 3D culture. Reversion of the T4-2 cells restored CAR to normal levels, as observed for many other parameters.40 Given the role of CAR as the primary receptor for adenovirus, the resistance of normal cells and the increased susceptibility of the T4-2 cells to adenoviral infection bode well for the development of adenoviral gene therapy vectors for the selective targeting of breast tumors in vivo.

Having established these paradigms with the HMT-3522 tumor progression series, we then investigated the role that some of these signaling and adhesion proteins played in the phenotype of a series of aggressive human breast carcinoma cell lines.49 The cell lines varied in their response to individual inhibitors but coordinate modulation of one or more of β1-integrin, MAPKK and PI3 Kinase was sufficient to induce phenotypic reversion or cell death in each case.

We have repeatedly shown that cells cultured in 3D behave differently to cells cultured on 2D plastic substrata, and that these cells recapitulate many phenotypic aspects of cells in vivo.40,42,50 The utility of this approach was demonstrated dramatically in our recent study on the role of cell polarity in sensitivity to apoptosis by chemotherapeutic agents. Nonmalignant and malignant cells are equally sensitive to apoptotic stimuli when cultured as monolayers. When nonmalignant and malignant cells were compared in both 2D and 3D, only the polarized 3D structures were resistant to a panel of apoptotic stimuli. When malignant cells were cultured in 3D, their disorganized, apolar colonies were sensitive to apoptosis, while the polar acinar structures they form upon reversion proved
resistant. This resistance correlated with both polarity and ECM composition and, crucially, was independent of genotype, growth rate or malignant status. Similarly, we have shown that while ERBB2 activation has the capacity to induce reinitiation of proliferation in growth-arrested mammary acini in culture, this ability is not shared with EGFR, even though either receptor could activate MAPK pathway signaling and induce proliferation of these cells on 2D plastic substrata. These unexpected differences could only be revealed using 3D models. The 3D IrBM model system is both physiologically relevant and amenable to genetic manipulation; these experiments establish a role for this assay in the identification of the factors required to correct the aberrations in individual tumors as well as those factors critical for the morphogenesis of the mammary gland in vivo. For a more detailed review, see Schmeichel and Bissell.

ENGINEERED MOUSE MODELS OF TUMOR REGRESSION

A number of systems have been described for the spatial and temporal regulation of transgene expression in experimental animals. Of these, the tetracycline (Tet) system of Gossen and Bujard has found favor because of its tight regulation of gene expression and because the inducing ligand, tetracycline or doxycycline, is itself innocuous even when administered for long periods of time. Consequently, unlike other systems, the Tet system is less prone to deleterious pleiotropic effects that can complicate the analysis of observed phenotypes. Since the original description of the Tet system, multiple improvements have been described. The application of these systems to transgenic mice has allowed the development of exquisitely controlled models of neoplasia initiated by numerous oncogenes in a variety of tissues. The ability to switch on or off the expression of an oncogene at will has facilitated experiments that address the requirements of tumors for continued expression of an initiating oncogene for the maintenance of the transformed state.

Early attempts to address these questions used tetracycline-dependent expression of the SV40 T antigen (TAg). TAg transforms cells by sequestration of both p53 and Rb, a phenomenon that has been comprehensively studied and reviewed. Conditional expression of TAg in the submandibular glands resulted in extensive ductal hyperplasia. This phenotype could be reversed by suppression of TAg expression, although the reversibility decreased with age of the mice, suggesting that the hyperplastic cells had accumulated further genetic or epigenetic changes that reduced their dependence on TAg expression.

The c-Myc protooncogene is overexpressed in a wide range of human tumors and many workers have analyzed the role of c-Myc deregulation in tumorigenesis. Targeted overexpression of an estradiol-inducible c-Myc-ER fusion protein in suprabasal keratinocytes led to increased proliferation, hyperplasia and papillomatosis in a ligand-dependent manner. This phenotype was reversible upon suppression of TAg expression. Similarly, conditional overexpression of c-Myc in lymphocytes yielded malignant T-cell lymphomas and acute myeloid leukemias. These phenotypes were reversed and normal hematopoiesis was restored upon suppression of transgene expression. This was associated with differentiation and apoptosis of the leukemic cells. Tumors that relapse in this model have typically acquired additional genomic alterations that allow c-Myc-independent growth. A small proportion of these mice had sustained activating Ras mutations, which allowed for Myc-independent growth.

The small G-protein, Ras, is a potent oncogene that is very commonly deregulated in many cancers, perhaps most strikingly in the pancreas, where 90% of tumors have activating Ras mutations. Overexpression of an activated Ras allele was sufficient to initiate melanoma formation in mice null for the INK4A tumor suppressor. Withdrawal of doxycycline led to the regression of these tumors to almost undetectable levels. Readministration of doxycycline led to a rapid relapse in this model. Tet system–regulated overexpression of Ras in the lung produced adenomas and adenocarcinomas after 2 months of transgene induction. Tumor formation was accelerated on both p53 and INK4A backgrounds. Switching off Ras expression promoted tumor regression by apoptosis. In a similar model, lung adenomas induced by FGF-10 overexpression regressed when FGF-10 expression was switched off.

The BCR-Abl fusion gene is formed by a balanced translocation between chromosomes 9 and 22 and is a common hallmark of chronic myelogenous leukemia (CML) and a subset of acute lymphoblastic leukemias (ALL). Tetracycline–regulated overexpression of the tyrosine kinase encoded by the BCR-Abl fusion gene induced acute B-cell leukemia with 100% penetrance. Suppression of oncogene expression led to the rapid normalization of the white blood cell count and complete regression of enlarged lymph nodes. Subsequent induction of BCR-Abl restored the leukemic phenotype, and these cycles of induction and regression could be reiterated multiple times in an individual animal. These experiments demonstrate the requirement for sustained BCR-Abl expression for the maintenance of the leukemic phenotype. Interestingly, one characteristic of BCR-Abl overexpressing CML cells is the loss of the ability to transduce growth inhibitory signals via β1-integrin from the bone marrow microenvironment. Downregulation of BCR-Abl expression using antistreptavidin nucleotides led to the restoration of integrin-mediated adhesion and a reduction in the proliferation rate. Restoration of integrin function was seen also upon treatment with interferon-α and with inhibitors of BCR-Abl kinase activity. Data from transgenic mouse models provide evidence for the importance of the integrin signaling pathways in mammary cancer. Overexpression of integrin-linked kinase (ILK) in the mammary gland induced a mild ductal and acinar mammary hyperplasia in young nulliparous mice, a proportion of which developed focal tumors, albeit with a long latency. Recent work using a conditional knockout of β1-integrin has shown that β1-integrin expression is required for tumorigenesis in mice overexpressing the polyoma middle T antigen in the mammary gland (Donald White and William Muller, personal communication).

The above approaches have been extended to investigate the degree to which metastases continue to depend on the oncogenic lesion that initiated the primary tumor. Tetracycline-dependent overexpression of an activated HER2/Neu allele in the mammary gland yields multiple mammary tumors with short latency and 100% penetrance. These tumors typically metastasize to the lung. Abrogation of Neu expression was sufficient to regress the primary tumors to the point where they were not palpably detectable. Interestingly, despite the number of genetic changes acquired during tumor progression, the pulmonary metastases were as dependent as the primary tumors on continued expression of HER2/Neu for their survival. These data provide further evidence that, even in advanced disease, the vast majority of cancer cells can still regress upon downmodulation of a single oncogene. However, unlike many similar studies, these experiments included a significant period of postregression follow-up. The majority of animals in which tumors had clinically regressed subsequently developed tumors that were Neu-independent, indicating that while most cells required Neu for maintenance of the transformed phenotype, a small proportion of cells in some tumors had acquired the ability to grow in a Neu-independent manner. Whether or not modula-
tion of additional pathways, as was done for metastatic cell lines in
3D cultures,49 could attenuate or kill the Neu-independent tumor
cells remains to be determined.

Collectively, these experiments suggest that the correction of 1
or 2 molecular defects may be sufficient to revert the malignant
phenotype, even when additional genetic alterations have been
acquired during tumor progression. While these data are undoub-
etedly striking, some concerns remain. In general, regression in these
studies is assessed by palpation and visual inspection. Often there
is no attempt to investigate the possibility of residual disease. As
shown by Moody et al.,75 a long period of postregression fol-
low-up can reveal latent residual disease that is missed in shorter
studies. A molecular analysis of such recurring tumors may shed
additional light on the mechanisms by which a tumor can escape
dependence on the expression of the initiating oncogene and
illuminate the signal transduction pathways and regulatory pro-
tein involved.

ACUTE PROMYELOCYTIC LEUKEMIA:
FROM BENCH TO BEDSIDE

The model of tumor reversion that has thus far yielded the
greatest clinical benefits is in the hematological malignancies. This
therapeutic paradigm is exemplified by the successful treatment of
acute promyelocytic leukemia (APL) by retinoic acid-based ther-
apies. The majority of cases of APL are caused by a chromosomal translocation that juxtaposes the genes encoding PML and retinoic
acid receptor α (RARα). PML–RARα binds retinoic acid-responsive
elements (RARE) in genomic DNA and recruits histone deacetylases,
resulting in a global repression of RARα target genes.76 The resultant block in myeloid differentiation leads to the
accumulation of promyelocytes.

Myeloid leukemia cells can be induced by a variety of cytokines
to differentiate into nondividing mature granulocytes and macro-
phages in culture and in vivo.77 All-trans retinoic acid (ATRA) is
now the regimen of first choice for treatment of APL, resulting in
the relocalization and degradation of the PML–RARα complex,
leading to the recognition of the initiating oncogene and
remission of APL in the majority of patients, while those who
relapse from the combined treatment can be successfully treated
with arsenic trioxide, ensuring a cure rate of up to 85% for this
disease.79

Successful therapy of APL has firmly established the paradigm
that differentiation therapy as a valid approach for the reversion of
malignancy in the treatment of neoplasia in human patients. It
remains the task of biomedical scientists and clinicians to identify
molecular targets for similar approaches in other human cancers.80

CONCLUSION

A critical question is often asked: Why pursue the phenotypic
reversion of malignancy? Surely it is better to look for more
efficient methods of killing tumor cells? Tumors are remarkable
creatures, possessed of manifold means to defeat the arsenal of
therapeutics arrayed against them. Among other things, the
 genome instability of tumors gives them a persistent evolutionary
advantage, ensuring the survival of stronger, fitter, more aggress-
sive cells that will go on to populate the body of their host. The
approaches that have been shown to extend the life of the tumor
are promising but clearly insufficient to eradicate the tumors.
In this sense, the microenvironment can be dominant over the malignant genotype.44
It is of course preferable to eradicate the tumor altogether, but
aggressive chemotherapy to eradicate a tumor often kills the host.
The malignant nature of tumors would indicate that multiple ap-
proaches may be necessary. This raises the possibility of the
long-term management of some cancers as a chronic condition in
which the malignant potential of the tumor cells is constrained, per-
haps for the lifetime of the patient.

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