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14. ABSTRACT To investigate breast cancer metastasis, we propose to use a tissue-specific viral delivery system that will allow the somatic delivery of secondary lesions in the background of a tumor-inducing primary oncogene. In the first year of support we established protocols for efficient in vitro cultivation and infection of mammary gland-derived normal, hyperplastic and tumorigenic cells. For the second year we started to analyze the behavior of mammary tumor cells when infected with candidate genes that are likely to promote invasion. We analyzed infected cells in mammary fat pad re-implantation assays and established a three dimensional basement membrane-based cell culture system for primary mouse mammary cells that will allow us to examine differences after infection of normal, hyperplastic, tumorigenic and invasive mammary cells in more detail.					
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Table of Contents

Introduction.....	4
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	9
References.....	10
Appendices.....	N/A

Introduction:

Several hallmarks of carcinogenesis, and of epithelial tumor progression in particular, have been identified during the past few decades. These critical features include uncontrolled proliferation, insensitivity to negative growth regulation, evasion of apoptosis, angiogenesis, and metastasis (1). Of all these steps, metastasis is probably the least understood for the simple reason that it is an elusive process to study: single invading cells can easily be mistaken for stromal cells, and migration to distant organs via lymphatics or blood vessels is hard to follow.

To investigate molecular players in breast cancer progression, we combined well characterized mouse breast cancer models expressing the primary oncogenes Neu or Wnt-1 with a tissue-specific viral delivery system (TVA system) that will allow the somatic delivery of secondary lesions. Multiple genes can be introduced and the cooperative action of these genes can be studied to see if they promote metastasis.

Report

During the first funding period we established protocols for efficient *in vitro* cultivation of mammary gland-derived normal, hyperplastic and tumorigenic cells, mainly from transgenic mice programmed to express *Wnt1* or *Neu* oncogenes and the avian virus subgroup A receptor, *TVA*, in the mammary gland. We have been able to infect cultivated cells at different stages of tumor progression with RCAS-A viruses and lentivirus (HIV) vectors pseudotyped with avian virus EnvA. Since the TVA infection-system produces only low levels of viral proteins, no infectious particles are formed and the virus is unable to spread. This leaves the viral receptor available for re-infection and allows introduction of several candidate genes and the study of cooperative action of these genes to promote tumor growth, invasion, and metastasis.

To begin to identify genes that can augment tumorigenesis in this model system, we have cloned different genes that have been suggested to promote late stage tumor progression and metastasis, including *TGF-beta1*, *Snail*, *Twist*, *amphiregulin*, and *CXCR4* (2-9) into RCAS and lentiviral vectors pseudotyped for EnvA. All of the RCAS viruses grow efficiently in DF1 cells, producing stocks that were concentrated to titers of 10^8 infectious particles per ml.

Viruses carrying different genes elicited different behaviors in mammary tumor cells on collagen coated plates. Figure 1 shows an example of cultivated *MMTV-TVA/MMTV-Neu* tumor cells, infected with RCAS viruses carrying markers and candidate progression factors. Cells infected with RCAS-*AP* or RCAS-*GFP* grow in a polarized manner, resembling cells in tumors from *MMTV-Neu* mice (Fig. 1A; 30-40% GFP-positive cells as estimated with fluorescence microscope); cells infected with RCAS-*Twist* show unpolarized patches (Fig. 1B); while cells infected with viruses encoding *Twist*, *Snail* and *Tgf-beta1* display more severe aberrant phenotypes, including single fibroblastoid-looking cells (Fig. 1C).

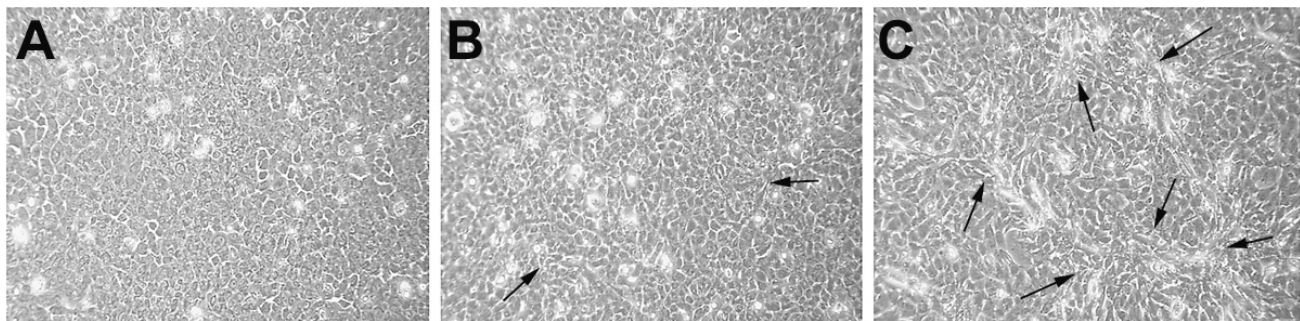


Figure 1 Growth phenotypes of infected *MMTV-Neu/MMTV-TVA* tumor cells. Tumor cells infected with RCAS viruses carrying *AP* and *GFP* (A); RCAS-*Twist* (B); RCAS viruses carrying *Twist*, *Snail* and *Tgfb1* (C). arrows indicate depolarized patches

Following infections of TVA-positive mammary tumor cells from different mouse strains (*MMTV-Neu* and *MMTV-Wnt1* transgenics), we are currently assessing the efficiency of tumor formation and enhanced metastasis to the lung after fat pad transplantation. In initial experiments, we placed cells infected RCAS viruses encoding one of the above mentioned tumor progression factors or GFP or alkaline phosphatase AP in cleared fat pads of Rag-/- mice. We let tumors grow until they reached 10% of mouse body weight and then surveyed lungs and other organs for metastases. All cultured, infected and replanted primary tumor cell populations were able to form tumors with similar latency and displayed visible metastases, including control infected cells that did only harbor control genes (GFP or AP). At this time, numbers of experimental mice that have been injected are not big enough to draw final conclusions. Moreover, each metastatic focus may represent a growth of cells unaffected by the infections or cells made more malignant by the infection. To judge this situation for each focus,

individual metastatic growths will be examined for proviral DNA by long distance PCR between viral priming sites, followed by cloning and identification of inserts by hybridization or nucleotide sequencing.

Because single genes may not be sufficient to augment invasive and metastatic properties on tumor cells (also indicated in the *in vitro* experiment shown in Figure 1B versus 1C), we are now introducing small sets of multiple viruses bearing different cDNAs, including candidate progression factors, known oncogenes, and reporter genes (such as *GFP*), into TVA-positive mammary cells.

At the same time, given the multitude of parameters and difficulties of the *in vivo* metastases experiments, we started to assay growth patterns and invasive behavior in three dimensional basement membrane extract gel assays. We established conditions for cultivation of mammary gland-derived normal, hyperplastic, and tumorigenic cells in three dimensional, basement membrane-extract gels. Experiments with epithelial cells grown in two dimensional cultures have severe limitations with respect to polarized morphology, specialized cell-cell contacts, and attachment to an underlying basement membrane. All of these features are necessary for the proper control of cellular proliferation, survival, differentiation and secretion (10). Cultivation of epithelial cells on a 2D planar surface impairs epithelial polarization, since nutrients and growth factors in the culture medium cannot pass the tight junctions of a fully polarized monolayer and fail to reach their basolaterally located receptors facing the plastic surface. These problems can be circumvented by culturing cells in three-dimensional (3D) collagen gel cultures, in which fully polarized cells form organotypic structures. These culture systems are able to recapitulate essential structural features of glandular epithelium and are amenable to experimental manipulation and detailed microscopic analyses. Furthermore, gels can be fixed, cut, and processed for immunohistochemistry and immunofluorescence.

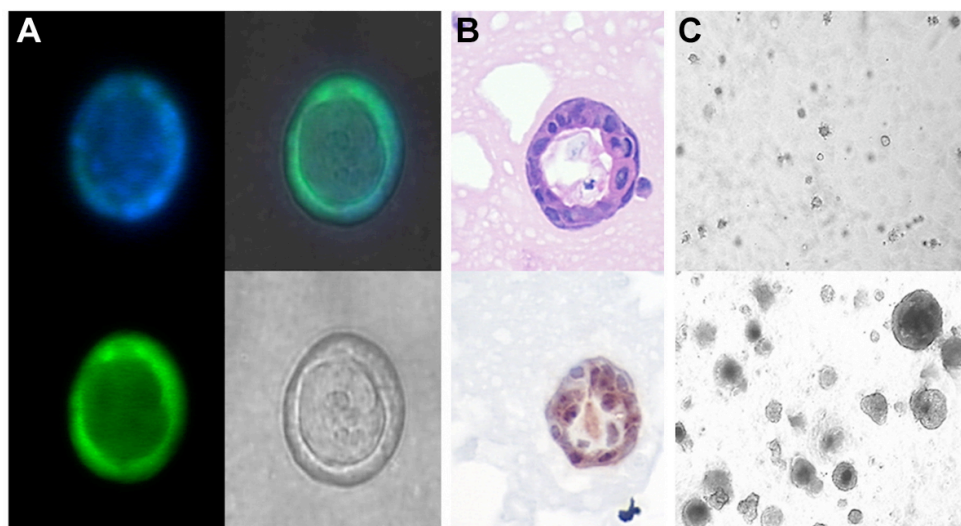


Figure 2. Organotypic growth of normal and tumorigenic primary mammary cells in 3D culture conditions **A.** Mammary gland cells harvested from a *beta-actin-GFP* transgenic mouse are forming acinar structures. Nuclei are stained with Hoechst 33342 (upper left); GFP expression can be observed in the gel (lower left); brightfield (lower right) and overlay (upper right) are shown. **B.** 3D gels can be

cut and stained for H&E (upper panel) and TVA (lower panel). **C.** Comparison of structures formed by normal mammary cells (small acini; upper panel) and Wnt1-expressing breast tumor cells (large solid structures; lower panel); both pictures taken under 2.5x magnification.

Cells derived from normal and hyperplastic mammary tissue grow in hollow acinar structures, while tumorigenic cells form enlarged solid balls (Fig. 2). These findings are in line with previously published results from other laboratories (4, 10-13). We also used RCAS virus vector carrying GFP in order to test whether infected tumor cells can still be grown in the 3D conditions and obtained solid green structures in part of the gels, allowing the use our delivery system for these experiments.

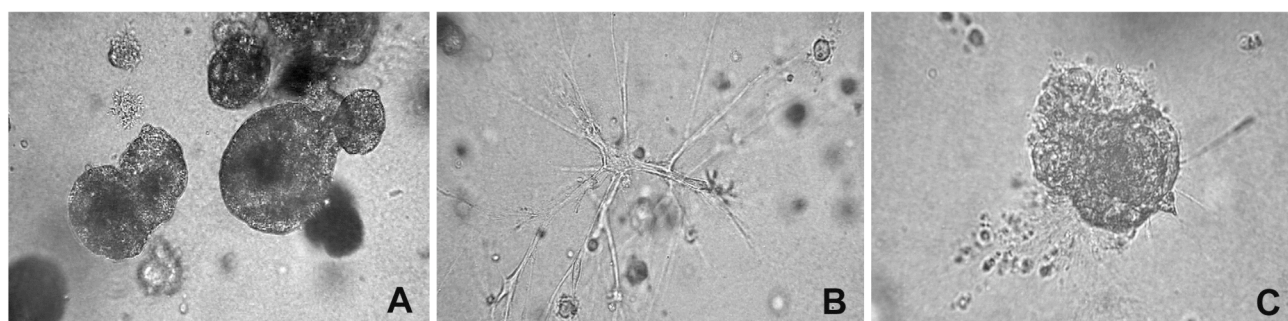


Figure 3. Growth of Wnt1 expressing tumorigenic primary mammary cells -treated or left untreated with TGFbeta- in 3D culture conditions **A.** Wnt1-expressing breast tumor cells grew into smooth surfaced, solid balls. 20x magnification **B.** When treated with 5 ng/ml TGFbeta for 10 days (starting 2 days after seeding), a subset of Wnt1-expressing breast tumor cells formed interconnected, elongated invasive structures. 20x magnification **C.** Another subset of seeded Wnt1-expressing breast tumor cells (TGFbeta treatment as described in 3B) grew more solid structures. In contrast to untreated control structures, these comparably smaller solid structures displayed rough edges that showed single sprouting cells. 40x magnification

Next we manipulated solid structures obtained from single tumor cells with soluble, activated TGFbeta to test invasive behavior in the three dimensional basement membrane extract gels. We observed a growth inhibitory effect on a large proportion of cells in the treated gels, which was not surprising, since TGFbeta1 is a potent growth inhibitor, with tumor-suppressing activity. However, cancers are often refractory to this growth inhibition either because of genetic loss of TGF-beta signaling components or, more commonly, because of downstream perturbation of the signaling pathway, such as by Ras activation (14). Indeed, a subpopulation of Wnt1-induced tumor cells formed elongated, presumably non polarized, invading structures (Figure 3B) that could not be seen in untreated control gels (excluding the possibility of remaining contaminating fibroblasts). Other surviving structures displayed cells on the surface that seem to sprout into the 3D matrix (Figure 3C).

To follow up these encouraging first steps, we seeded the gels with mammary cells derived from trigenic *TetO-Myc/TetO-Kras^{G12D}/MMTVrtTA* mice, that allow regulation of *Myc* and *Kras^{G12D}* oncogenes by administration of doxycycline. As expected, uninduced cells form hollow acinar structures, similar to structures formed by cells derived from wild type animals (Fig.4A,D). When induced in culture with doxycycline (1mg/ml) to overexpress *Myc* and mutant *Kras*, these structures grew into solid balls within 4 days (Fig.4B,E). When we elute doxycycline from the gels by multiple washes with media, growth ceases and the inner region of the solid clusters of cells clears, in a fashion similar to the formation of acinar structures described in (15, 16).

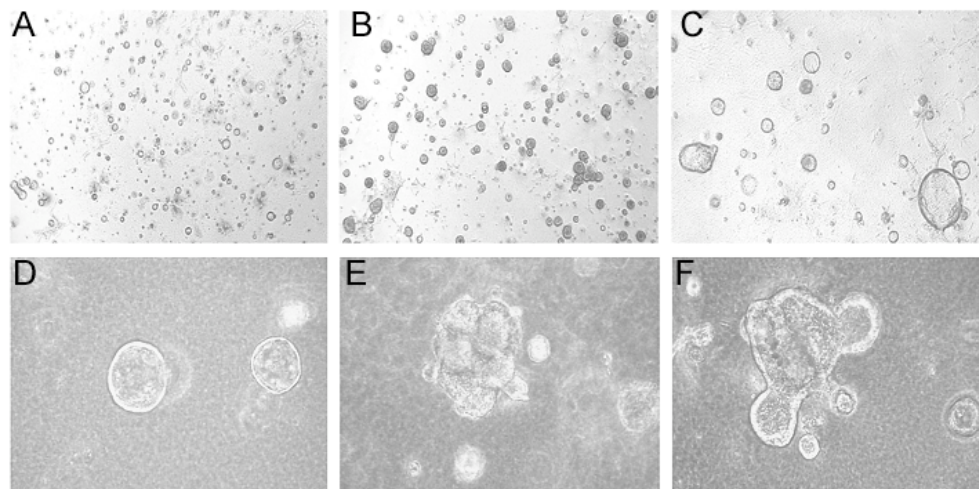


Figure 4. Regulation of transformation in primary mammary cells with inducible Myc and mutant Kras
Appearance of primary 3D cultures from previously uninduced trigenic animals before transgenes induction (**A,D**), after 4 days of induction in culture (**B,E**), and 4 days after doxycycline removal (**C,F**); **A, B, C** 2.5x; **D, E, F** 20x

An outermost layer of cells remains viable and retains the irregular shape assumed during doxycycline stimulation (Fig.4C,F). Induced solid structures maintained on doxycycline during this period (10 days) did not show any changes in the inner mass of cells. When analyzed in more detail employing timelapse microscopy, the cells stop proliferating and collapse in the inner mass of the solid structures around 20 hours after doxycycline withdrawal.

We have crossed MMTV-TVA mice into trigenic *TetO-Myc/TetO-Kras^{G12D}/MMTVrtTA* mice in order to use the ALV delivery system in the context of regulatable oncogenes.

To enlarge the cohort of factors to be tested in the *in vivo* and *in vitro* experiments outlined above, we are guided by published gene expression profiling studies that have identified upregulated genes that may play a role in metastasis (11, 17-23). Special attention is given to genes identified by J. Massague and his colleagues as metastasis-promoting genes in human breast cancer cell lines (e.g. MDA-MB-231 cells) and implicated in tissue-specific metastatic behavior in their subsequent studies (18, 19, 24, 25). Some of those genes include receptors (*CXCR4*, bone metastasis gene signature (BoMS), *VCAM1*, *IL13Ra2* lung metastasis gene signature (LMS)), matrix metalloproteases (*MMP1* (BoMS), *MMP1* and *MMP2* (LMS)), transcription factors (*Id1* and *Id3*), and secreted factors (*IL11* (BoMS), *epiregulin*, *CXCL1*, *angiopoietin-like 4*, *tenascin C* (LMS)). Further, we will give careful attention to the list of genes recently found to be mutated in human breast cancer cell lines (and confirmed by sequencing of primary human breast cancers) by Sjoblom et al (26). Many of these genes are not recognized “cancer genes” and have potential roles in tumor progression because they encode proteins are likely to affect cell motility, structure, or adhesiveness (e.g. *Filamin B*, *Myosin*, *Spectrin*, *Tectorin- α* , *Cadherin 20*, *ADAM metalloproteinase domain 12*).

To help us choose among the many candidate genes described in these several studies, I am working with the Computational Biology Center run by Dr. Chris Sander at MSKCC to narrow down the most promising candidates.

Key Research Accomplishments

.) Mammary tumor cells infected with different genes that have been proven to promote late stage tumor progression and metastasis (*TGFβ*, *snail*, *twist*) show different morphologies when grown on collagen 1 as compared to cells with control vectors.

.) We established conditions for cultivation of mammary gland-derived normal, hyperplastic, and tumorigenic cells in three dimensional, basement membrane-extract gels.

.) We are able to manipulate solid structures obtained from single tumor cells with soluble, activated TGFbeta to see invasive behavior in the three dimensional basement membrane extract gels.

.) We can grow mammary cells derived from trigenic *TetO-Myc/TetO-Kras^{G12D}/MMTVrtTA* mice, that allow regulation of *Myc* and *Kras^{G12D}* oncogenes by administration of doxycycline, in the three dimensional basement membrane extract gels. We see changes in the phenotype of the grown organoids according to their status of doxycycline treatment.

Reportable Outcomes

None

Conclusions

.) The *in vitro* experiment examining the effects of single and multiple infected mammary tumor cells (Figure 1) and preliminary results of our transplanted mammary tumor cells infected with just one type of RCAS vector suggest that delivery of multiple cooperating lesions is an important factor for enhancing metastases. We are currently working on analyses of cells infected with small sets of vectors, including candidate progression factors, known oncogenes, and reporter genes.

.) We established a three dimensional basement membrane based cell culture system for primary mouse mammary cells that will allow us to examine differences in normal, hyperplastic, tumorigenic and invasive behavior of infected mammary cells in more detail.

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