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14. ABSTRACT The purpose of this funded research program is to examine different modes of movement used by mammary tumor cells during local invasion and metastasis. In year 2 of the program I have expanded upon the role of Rho /ROCK signaling in driving tumor cell migration and invasion. Specifically, I have found that collagen I, a component of aggressive primary human breast tumors, promotes an invasive phenotype in a 3D culture system. Collagen I promotes invasion through an increase in actomyosin contractility in MMTV-neu-derived mouse mammary tumor cells. Interestingly, a highly contractile phenotype is normally restricted to myoepithelial/basal cells of the mammary gland, as well as the more aggressive basal-like breast cancers. I have experimental evidence, therefore, that collagen I may be promoting a more aggressive basal clinical subtype in primary breast tumors. In addition, I have found that MMTV-neu-derived cells express markers of the epithelial-mesenchymal transition (EMT), yet are non-invasive in Matrigel reconstituted basement membrane. In the presence of collagen I, however, the cells acquire a highly motile mesenchymal phenotype. These results suggest that the invasive properties of mammary tumors depends on the extracellular matrix environment, through an impact on Rho/ROCK signaling.					
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INTRODUCTION

The purpose of this funded research program is to examine different modes of movement used by mammary tumor cells during local invasion and metastasis. The host laboratory and another group previously identified two alternative forms of movement—the classic, elongated or mesenchymal type of movement, and a more rounded or amoeboid type of movement (Sahai and Marshall, 2003; Wolf et al., 2003). When the mesenchymal form of movement is blocked in cultured melanoma and fibrosarcoma cells, the cells compensate by acquiring the rounded or amoeboid type of movement. These observations suggest that tumor cells can adopt alternative strategies for invasion of adjacent tissue and metastasis in vivo. This hypothesis has important clinical implications for blocking the metastatic spread of breast tumors. The mesenchymal type of invasion, for example, has been shown to require pericellular proteolysis of the basement membrane. The rounded or amoeboid form of movement, however, has been shown to be independent of proteolytic activity (Wolf et al., 2003). The disappointingly low efficacy of proteolysis inhibitors in clinical trials, therefore, may be due to a compensatory switch from the proteolysis-dependent elongated form to the proteolysis-independent rounded form of migration and invasion (Coussens et al., 2002).

Although the ability to switch modes of movement reveals an important property of tumor cells in vitro, it is not known if this phenomenon occurs in invasive mammary tumors in vivo. The experiments outlined in this funded research program, therefore, were designed to elucidate the modes of mammary tumor cell movement in mouse models of mammary gland tumorigenesis. In addition, the work was designed to address the role of the Rho family of small GTPases in regulating cell morphology, migration and invasion in vivo. Rho family members Rho and Rac1 have previously been shown to drive rounded and mesenchymal tumor cell movement, respectively (Sahai and Marshall, 2003). By investigating the modes and mechanisms of tumor cell movement in mammary tumors, this funded program of work was designed to reveal more effective therapies for blocking the metastatic dissemination of mammary tumor cells in vivo.

BODY OF ANNUAL REPORT MONTHS 1-12

Months 1-12 (Task 1) of the original Statement of Work (SOW) involved analysis of mouse mammary tumor cell movement in 3-D collagen gels and in vivo. The analysis was to be carried out on primary tumor cell explants from the MMTV-erbB2/HER2/neu (MMTV-neu) and BRCA-1/- mouse models of mammary tumorigenesis (Guy et al., 1992; McCarthy et al., 2007). The movement of MMTV-neu and BRCA-1/- mouse mammary tumor cells was characterized in 3-D culture by time-lapse videomicroscopy. Bright field time-lapse videomicroscopy revealed that the MMTV-neu cells moved as both rounded and elongated cells on a thick bed of fibrillar type 1 collagen (Figure 1A shows a still image taken from a time-lapse video of MMTV-neu cells on a thick bed of type 1 collagen). This result demonstrates the plasticity of these cells in terms of morphology and movement, and suggests that they

may be able to utilize different modes of movement in vivo. MMTV-neu mice therefore represent candidate models for the analysis of different modes of mammary tumor cell movement and invasion in vivo. In addition to MMTV-neu cells, cells from BRCA-1^{-/-} derived mouse mammary tumors also exhibit both contractile and mesenchymal behavior on a collagen gel (Figure 1B). Due to difficulty in maintaining BRCA-1^{-/-} cells in culture, however, the experimental results from year 1 were generated primarily from MMTV-neu derived cells. Attempts to analyze the movement of BRCA-1^{-/-} cells will nonetheless continue in year 2.

Task 1 includes labelling of tumor cells with fluorescent labels for in vivo imaging. Labelling facilitates future in vivo imaging of tumor cell morphology and movement using two-photon intravital confocal microscopy. Fluorescence labelling of tumor cells from MMTV-neu mice was accomplished by transduction with a lentiviral vector expressing green fluorescent protein (GFP) and the puromycin resistance gene (vector purchased from Open Biosystems) (Figure 2A). The GFP-labelled cells were subsequently transplanted into the mammary fat pads of immunocompromised nude mice. Mammary tumors were excised after two weeks of growth and imaged using a standard confocal microscope (Figure 2B). In addition to the primary tumor, lungs were resected from tumor-bearing animals and imaged under a confocal microscope (Figure 2C). The combined results of these experiments demonstrates the feasibility of in vivo imaging of GFP-labelled mouse mammary tumor cells, both in primary tumors and in metastatic target sites such as lung. As described in Task 1(C), this intravital imaging technique is to be used for determining the modes of movement used by mouse mammary tumor cells during local invasion and metastasis in vivo. The imaging of tumor cell movement in vivo was not attempted in year 1, but is currently underway with the assistance of collaborators listed in the original proposal. Intravital imaging of tumor cell movement will subsequently be used for analyzing the impact of Rho and Rac1 signalling on cell shape and movement in vivo in years 2 and 3 of the program. Similarly, intravital whole-animal imaging of metastatic dissemination (Task 1(d)) has been moved from year 1 to years 2 and 3 of the program.

Year 2 of the program (Task 2) involved experiments to address the roles of Rho kinase (ROCK—a downstream Rho effector) and Rac1 in mammary tumor cell morphology and movement. ROCK activity and actomyosin contractility have previously been shown to drive rounded cell morphology and amoeboid movement in melanoma and fibrosarcoma cells (Sahai and Marshall, 2003). Rac1 activity, in contrast, has been shown to be associated with elongated or mesenchymal movement (Sahai and Marshall, 2003). Although the role of these proteins in mammary tumor cell movement was to be addressed in year 2, several important experiments have been performed ahead of schedule. The first of these experiments involved the application of a chemical inhibitor of ROCK to the MMTV-neu cells on a thick collagen gel. The application of this inhibitor (Y27632) resulted in impaired locomotion of the rounded cells, consistent with the role of ROCK in driving amoeboid movement in other tumor cell types (Sahai and Marshall, 2003) (data not shown).

In contrast, the application of a Rac1 inhibitor had no effect on the elongated movement of MMTV-neu cells on collagen (data not shown). This

may be explained by the limited specificity of this inhibitor, which targets only one type of guanine nucleotide exchange factor (GEF) upstream of Rac1. As a result, other Rac1 GEF proteins, such as DOCK180, may still be active in this assay. As an alternative approach to inhibiting Rac1 activity (as proposed in Task2), we obtained short-hairpin RNA (shRNA)-expressing lentivirus vectors targeting Rac1 (Open Biosystems). Stable expression of the shRNA sequences was accomplished by selection with puromycin, due to the inclusion of a puromycin-resistance gene in the vector. MMTV-neu cells stably expressing three independent shRNA constructs targeting Rac1 exhibited efficient knock-down of Rac1 at the protein level (Knock-down efficiency of Rac1 for two shRNA constructs are shown in Figure 3). Corresponding reduction in phospho PAK, a Rac1 effector, is also shown in Figure 3). Preliminary analysis of cell movement in Rac1 knock-down cells revealed an impairment of elongated movement on type 1 collagen, consistent with the role of Rac1 in promoting mesenchymal movement in other cell types (Sahai and Marshall, 2003) (data not shown).

During the course of these experiments, we made additional observations which may have important implications for our understanding of mammary tumor cell shape and movement. Importantly, we have found that cell shape and modes of movement and invasion are driven by the composition of the extracellular matrix (ECM), which may impact on Rho and Rac1 signalling. Cells on a thick bed of laminin-rich Matrigel™, for example, exhibit a rounded, highly contractile phenotype (Figure 4A). In the presence of fibrillar type 1 collagen, however, a large proportion of the cells acquire an elongated, mesenchymal morphology (Figure 4B). Upon closer examination, we have observed tumor cells aligned in the direction of stress fibres in the collagen gels (Figure 5). In this regard, it is interesting to note that tumor cells have been observed invading local tissue by migrating along fibrillar collagen in vivo (Provenzano et al., 2006). We are starting to investigate, therefore, whether fibrillar collagen provides a novel mechanism for driving rounded and elongated movement and invasion in vivo. We are particularly interested in determining if Rho-driven rounded movement and Rac1-driven elongated movement are regulated by the local ECM composition at the tumor-stroma interface. These observations provide an interesting addition to the experiments and aims described in the original proposal.

BODY OF ANNUAL REPORT MONTHS 13-24

As described in the first annual report above (months 1-12), we have characterized the roles of Rac1 and Rho/ROCK signalling in driving tumor cell shape and motility in 3D collagen gels. This aspect of the proposal was described in Task 2 (months 13-20) of the original SOW, which also included in vivo analysis of tumor cell movement using 2-photon microscopy. Preliminary analysis involving immunohistochemistry and western blot, however, revealed little evidence Rho/ROCK activity in mammary tumors from MMTV-neu mice (data not shown). In addition, Rho/ROCK activity did not increase upon inhibition of Rac1 expression in these tumors (data not shown). As a result, we were unable to detect with confidence any changes in tumor

cell shape upon inhibition of Rac1 or Rho/ROCK activity in vivo (data not shown).

Careful analysis of the mouse mammary tumors, however, did reveal a striking molecular phenomenon which was consistent with observations made in the 3D cell culture system. As described in the previous annual report above, we observed an increase in mesenchymal properties of MMTV-neu-derived tumor cells in the presence of collagen I (Figure 4B). In addition, we showed evidence that the tumor cells were migrating parallel to the direction of lines of contraction in the collagen I gel (Figure 5). In the past year (months 13-24), we have generated additional data confirming that the addition of collagen I induces a highly contractile phenotype in the MMTV-neu-derived tumor cells, as evidenced by an increase in myosin light chain (MLC) phosphorylation and contraction of the collagen I gel (Figure 6A-C). The role of Rho/ROCK-induced actomyosin contractility in contraction of the collagen I gel was confirmed by application of a Rho kinase inhibitor, Y27632 (Figure 6D).

The induction of actomyosin contractility in our system is consistent with reports that matrices of high elastic index, such as collagen I, can induce Rho/ROCK activity in cultured cells. As a result, we wanted to determine if actomyosin contractility was associated with a more rigid tumor microenvironment in vivo. Using an antibody to the phosphorylated form of MLC, we indeed detected elevated levels of phospho-MLC in regions of MMTV-neu tumors which were adjacent to more dense regions of extracellular matrix (ECM) (Figure 7A). In contrast, no phospho-MLC could be detected when the tumor was embedded in the much softer environment of the fat-rich mammary fat pad (Figure 7B).

To further understand the significance of these observations, we characterized the normal mouse mammary gland with regards to contractile properties. As shown in Figure 7, contractility associated with MLC phosphorylation is normally restricted to the contractile myoepithelial/basal layer of the mammary gland epithelium (Figure 7C). In contrast, the luminal epithelial cells show no evidence of phospho-MLC (Figure 7C). We then applied the phospho-MLC antibody to sections of mouse mammary tumor from the Brca1^{-/-}; p53^{+/-} mouse model. As described in the original proposal and SOW, the Brca1^{-/-}; p53^{+/-} mouse model represents a model of basal cell mammary carcinoma. As shown in Figure 7, tumor cells in these mice express high levels of phosphorylated MLC, consistent with the contractile properties of the basal-like cells from which they are derived (Figure 7D). Combined with the results of the 3D cell culture model, these observations suggest that a more rigid tumor matrix rich in collagen I may induce a more contractile, basal-like phenotype in luminally-derived tumors in MMTV-neu mice. Since basal-like tumors are a more aggressive type of human breast cancer, these results may have important implications for understanding the etiology of aggressive basal-like human cancer. Further exploration of this phenomenon may therefore lead to more effective prognostic criteria and treatment of human breast cancer.

Another important observation arising from this funded program concerns the relationship between the stromal matrix and the genetic program of the mammary tumor cells. Specifically, we found that cells in MatrigelTM express markers of a luminal epithelial origin (E-cadherin and cytokeratin 18),

as well as markers of the more invasive mesenchymal phenotype (N-cadherin, vimentin) (Figure 8). In Matrigel™, however, the cells showed no evidence of an invasive phenotype. In contrast, when collagen I was presented to the cells they acquired a mesenchymal morphology and were highly invasive (Figure 4). These results are consistent with the observation that tumor cells expressing EMT markers in situ are non-invasive in a fatty stroma, as compared to the same cells in a more dense tissue matrix (compare Figure 8E and 8F). These results provide evidence that the stromal matrix may be dominant over the genetic program of the mammary tumor cells. From a clinical perspective, these observations suggest that the onset of an aggressive, mesenchymal-like tumor phenotype can be predicted more accurately from a combination of tumor cell signature and the composition of the tumor stroma.

In summary, our work remains focused on the role of cell shape in metastatic disease progression. In the original proposal, we hypothesized that intrinsic Rac1 and Rho/ROCK signalling would dictate the mode of movement used by the mammary tumor cells in vivo. What we have found, however, is evidence of a much more intricate balance between the extracellular matrix (ECM) and the induction of invasive cellular properties, through an impact on actomyosin contractility. Rather than involving the entire tumor, as hypothesized in the original proposal, we have evidence that the relationship between the ECM and invasive cellular properties may be restricted to regions of the tumor rich in fibrillar ECM proteins such as collagen I. In addition, we have experimental evidence that the tissue stroma can determine the invasive phenotype of tumor cells that harbour an EMT-like genetic profile. These results may have important implications for prognosis and treatment of aggressive breast cancer.

With regards to personal training landmarks, I have acquired important new skills and knowledge during the first year of this funded program. After working with Professor Christopher Marshall in the host laboratory I now have a deeper understanding of the intricacies of cell signalling, and the role that cell signalling pathways play in tumor cell behavior. Working with 3-D gels and time-lapse videomicroscopy I have also gained an appreciation of the way in which mammary tumor cells interact with and respond to the matrix microenvironment. My exposure to more detailed issues of cell biology has enhanced my understanding of mammary tumor invasion and metastasis in the mouse models with which I have previous experience. In addition, I have been working with the histopathology unit of the Institute of Cancer Research to analyse the relationship between tumor stroma and the expression of molecular markers within the tumor cells. The combination of cell biology, cell signalling and histopathology skills, with my extensive experience in mouse models will undoubtedly strengthen my ability to perform meaningful research into breast cancer metastasis in the future.

KEY RESEARCH ACCOMPLISHMENTS (year 1)

- cultured primary tumor cells from MMTV-neu and BRCA-1/- mouse models of mammary gland tumorigenesis

- characterized morphology and movement on thick collagen gels : confirmed that MMTV-neu and BRCA-1-/- mammary tumor cells use both rounded (amoeboid) and elongated (mesenchymal) forms of movement
- induced efficient knock-down of Rac1 in MMTV-neu cells through stable expression of short-hairpin RNA (shRNA) against Rac1 sequences
- demonstrated that rounded and elongated movement of MMTV-neu cells requires Rho kinase (ROCK) and Rac1 activity, respectively
- demonstrated that elongated movement may be driven by fibrillar collagen
- demonstrated feasibility of imaging GFP-labelled MMTV-neu cells in vivo

KEY RESEARCH ACCOMPLISHMENTS (year 2)

- showed that collagen I induces myosin light chain (MLC) phosphorylation and actomyosin contractility in MMTV-neu tumor cells
- showed that MLC phosphorylation correlates with a dense tumor tissue microenvironment in MMTV-neu mouse mammary tumors in vivo
- showed that increased MLC phosphorylation in collagen I is characteristic of a more aggressive basal-like tumor cell phenotype
- showed that the invasive phenotype of cells expressing EMT-like markers depends on the presence of collagen I in the extracellular matrix

REPORTABLE OUTCOMES

Abstract: Era of Hope Meeting, Baltimore, MD, June 25-28, 2008:

The dissemination of breast cancer cells from a primary lesion to distant sites such as brain or bone presents the most serious threat to patient survival. As part of the metastatic process, cells must migrate from the primary tumor and invade into adjacent tissue and across the endothelial layer of blood vessels. The way in which tumor cells navigate through the fibrous extracellular matrix (ECM), however, is not clear. One proposed mechanism involves the loss of epithelial properties and the acquisition of a more motile, fibroblast (or mesenchymal)-like cell. These mesenchymal-like cells are characterized by an elongated, rather than cuboidal, morphology, as well as prominent actin-rich protrusions at the leading edge of the cell. Adhesive forces at the front of the cell, together with co-ordinated waves of actomyosin contraction and retraction of the trailing edge, drives forward migration of these cells. In addition, the foremost membrane protrusions are often rich in protease activity which facilitates invasion by degrading the ECM at the front of the cell. Until

recently, this epithelial-mesenchymal transition (EMT) was generally regarded as the predominant mechanism of tumor cell migration and invasion. Recent reports, however, describe an alternative form of cell movement in which the cells acquire a rounded or amoeboid morphology, allowing them to squeeze through interstitial spaces of the ECM. This form of movement is of great clinical interest, since it circumvents the need for pericellular proteolysis and may therefore complicate our ability to block metastasis using protease inhibitors. Importantly, it has been shown that various tumor cell lines have the capacity to switch from the elongated to the amoeboid form of movement in the presence of protease inhibitor cocktails in culture. This mesenchymal-amoeboid transition (MAT) was shown to involve downregulation of Rac1 activity, which would normally drive formation of membrane protrusions through the assembly of a branched actin network at the leading edge of the cell. In turn, activation of the Rho kinases ROCK1 and ROCK2 leads to high levels of actomyosin contractility, driving formation of the highly contractile, rounded morphology. Although this phenomenon has been demonstrated in cell culture, it is not known if the MAT can occur in vivo. The purpose of our proposed project, therefore, is to determine the types of movement employed by tumor cells in two well established mouse models of human breast cancer. Mammary tumors in these models are driven by expression of the activated *erbB-2/neu* oncogene, or by loss of the *BRCA-1* tumor suppressor. Analysis of explanted cells from these tumors reveals the capacity to use either elongated or rounded forms of movement ex vivo, depending on the substratum. The use of pharmacological inhibitors confirms that these forms of movement require Rac1 and Rho/ROCK activity, respectively. Using a lentivirus-shRNA approach, we are currently targeting these pathways in vivo to determine the impact on local invasion and metastasis, and to determine whether one pathway can compensate for cell movement following loss of the other pathway in vivo. We hope that this work will lead to a more detailed understanding of how modes of tumor cell movement in vivo contribute to breast cancer progression, thereby identifying more effective prognostic markers and therapeutic targets for this devastating disease.

CONCLUSION

Several important experimental observations were generated in year 2 of the funded research program. These observations were not predicted in the hypotheses in the original proposal, but may have clinical importance for breast cancer treatment and for predicting patient outcome. Specifically, it was shown that collagen I, which accumulates in aggressive disease, induces a highly contractile phenotype in MMTV-neu mouse mammary tumor cells. High contractility is normally restricted to myoepithelial/basal cells in the normal gland, as well as basal-like tumors from the *Brca1*^{-/-}; *p53*^{+/+} mouse model. Since basal-like breast cancers are more aggressive than other clinical subtypes, these observations may have important implications for understanding how collagen I accumulation contributes to poor patient outcome.

In addition, it was shown that MMTV-neu-derived tumor cells do not invade into Matrigel™ reconstituted basement membrane, even though they

express markers of the epithelial-mesenchymal transition (EMT). In the presence of collagen I, however, the cells acquired a highly motile and invasive phenotype, suggesting that the tumor stroma may be a determining factor as to whether mesenchymal-like tumor cells become invasive in vivo.

Together, the results of years 1 and 2 of the research program suggest that the phenotype of invasive breast cancers is driven by the extracellular matrix, through an impact on Rho/ROCK signaling and actomyosin contractility. These results have important clinical implications for blocking the spread of metastatic breast cancer, and for predicting the risk of developing aggressive disease.

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APPENDICES

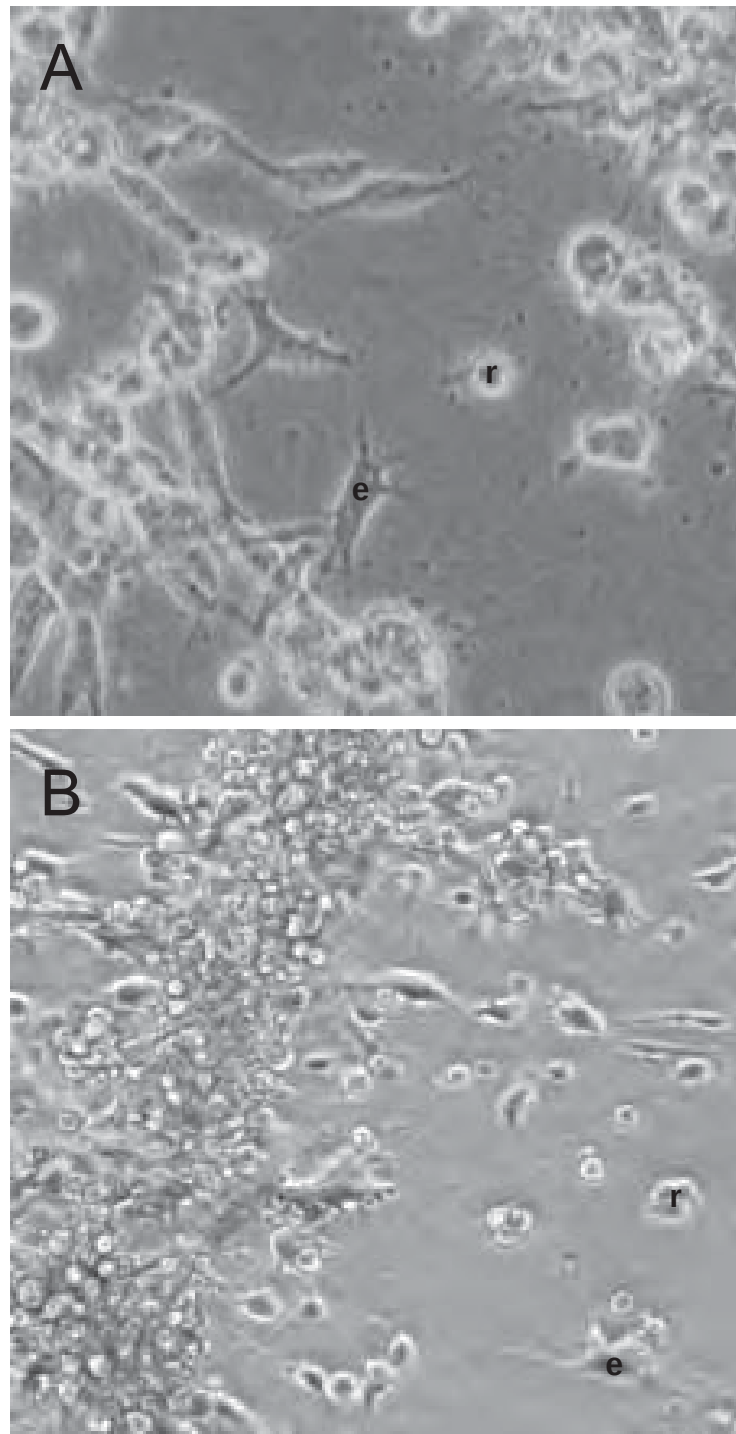


Figure 1

Primary mouse mammary tumor cells utilize both rounded and elongated forms of movement ex vivo. (A) Still image from time-lapse video microscopy of MMTV-neu-derived mouse mammary tumor cells, showing both rounded (r) and elongated (e) morphologies on a thick gel of type 1 collagen. (B) The same analysis repeated for BRCA-1^{-/-} mouse mammary tumor cells, again showing both rounded and elongated cells.

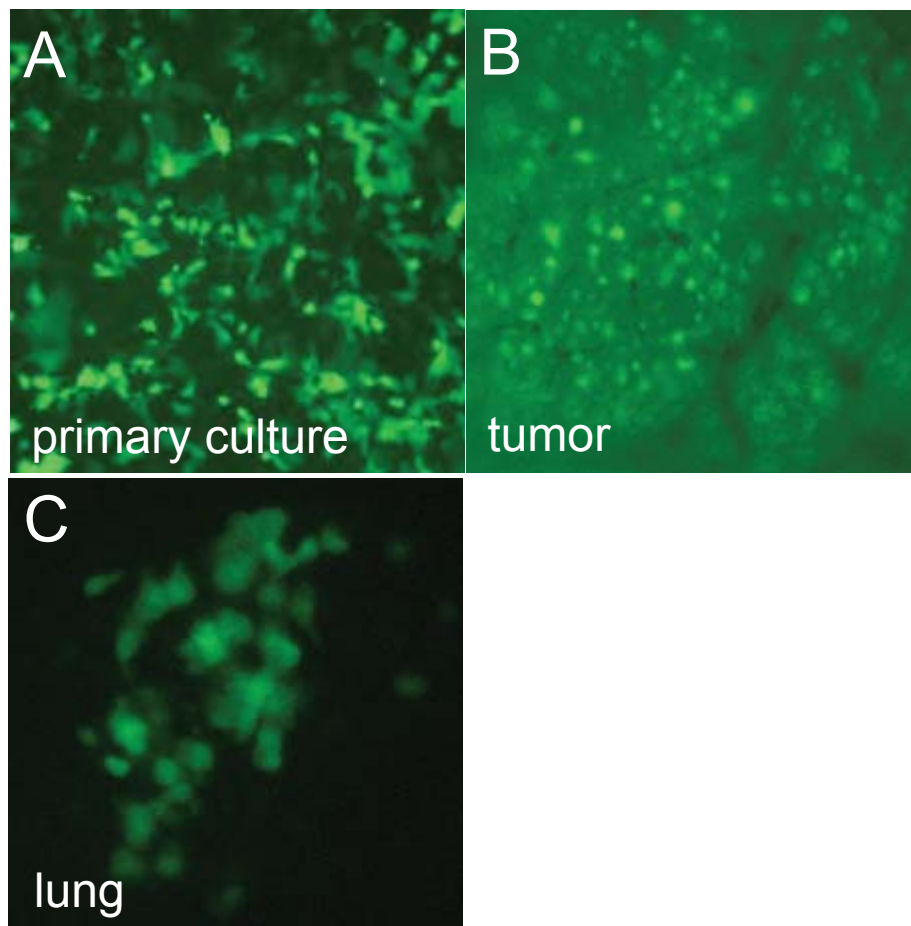


Figure 2

MMTV-neu-derived mouse mammary tumor cells expressing green fluorescent protein (GFP) can be imaged in vivo. (A) Monolayer culture of MMTV-neu cells stably expressing GFP. (B) Confocal image of mouse mammary tumor 3 weeks after transplantation of GFP-expressing MMTV-neu cells into mammary fat pad. (C) Disseminated GFP-expressing cells can be detected in the lungs of animals following mammary tumor growth at site of transplantation.

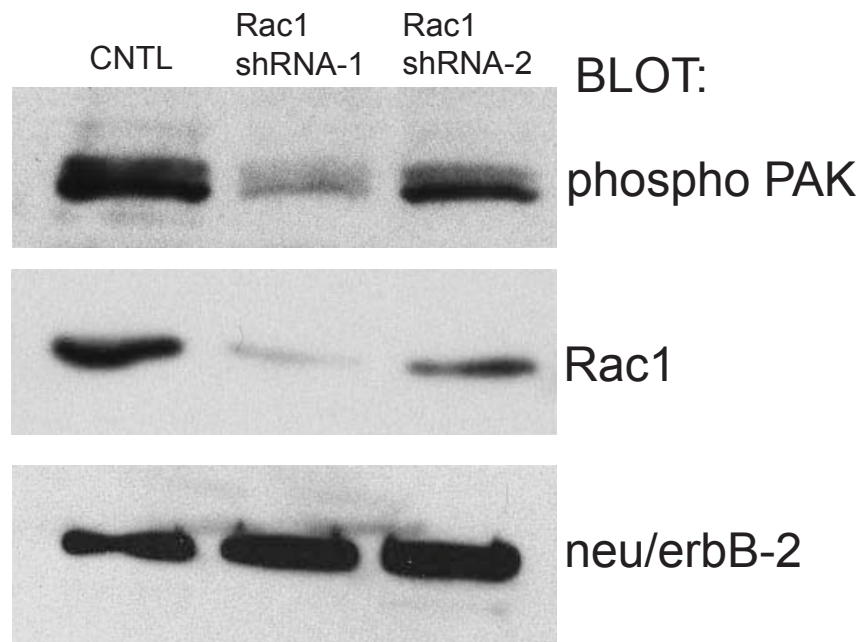


Figure 3

Rac1 expression can be knocked down in MMTV-neu cells by stable expression of short hairpin RNA (shRNA) targeting Rac1 sequences. The immunoblot shows the level of Rac1 knock down using 2 independent shRNA constructs (middle panel), as well as the corresponding reduction in phospho PAK, a Rac1 effector (top panel). Total neu/erbB-2 levels are used as an internal loading control (bottom panel).

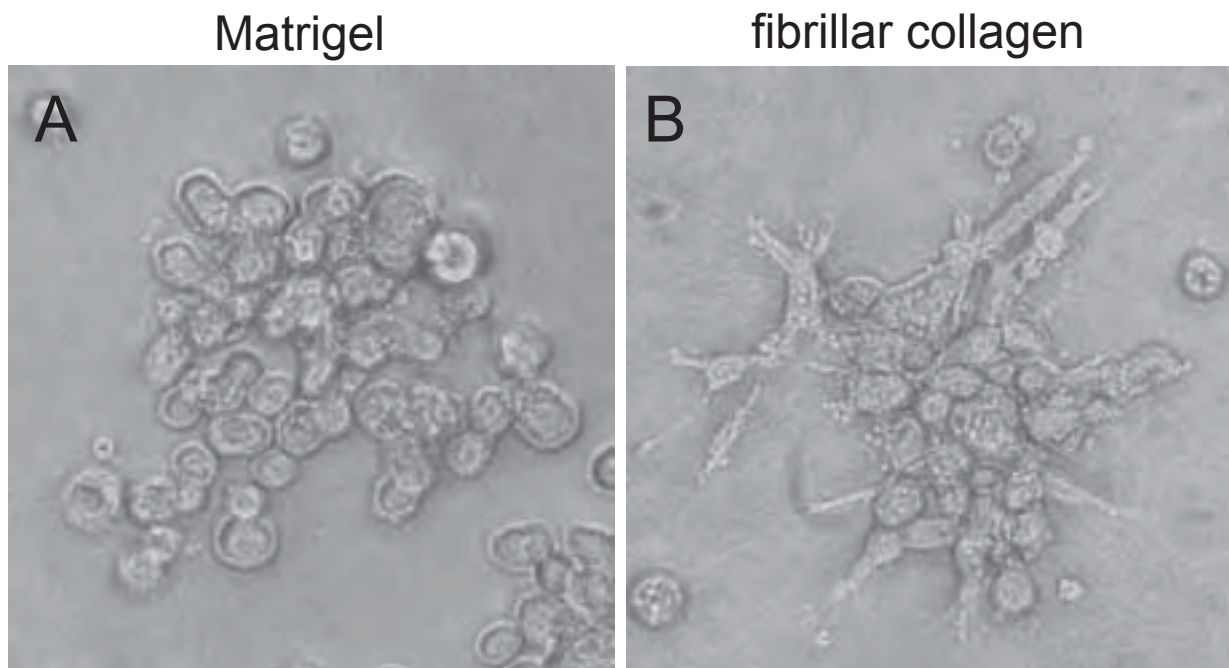


Figure 4

Cell shape is dictated by the extracellular matrix composition.

(A) MMTV-neu mouse mammary tumor cells are rounded on laminin-rich Matrigel.

(B) MMTV-neu cells acquire a mesenchymal phenotype on fibrillar type 1 collagen.

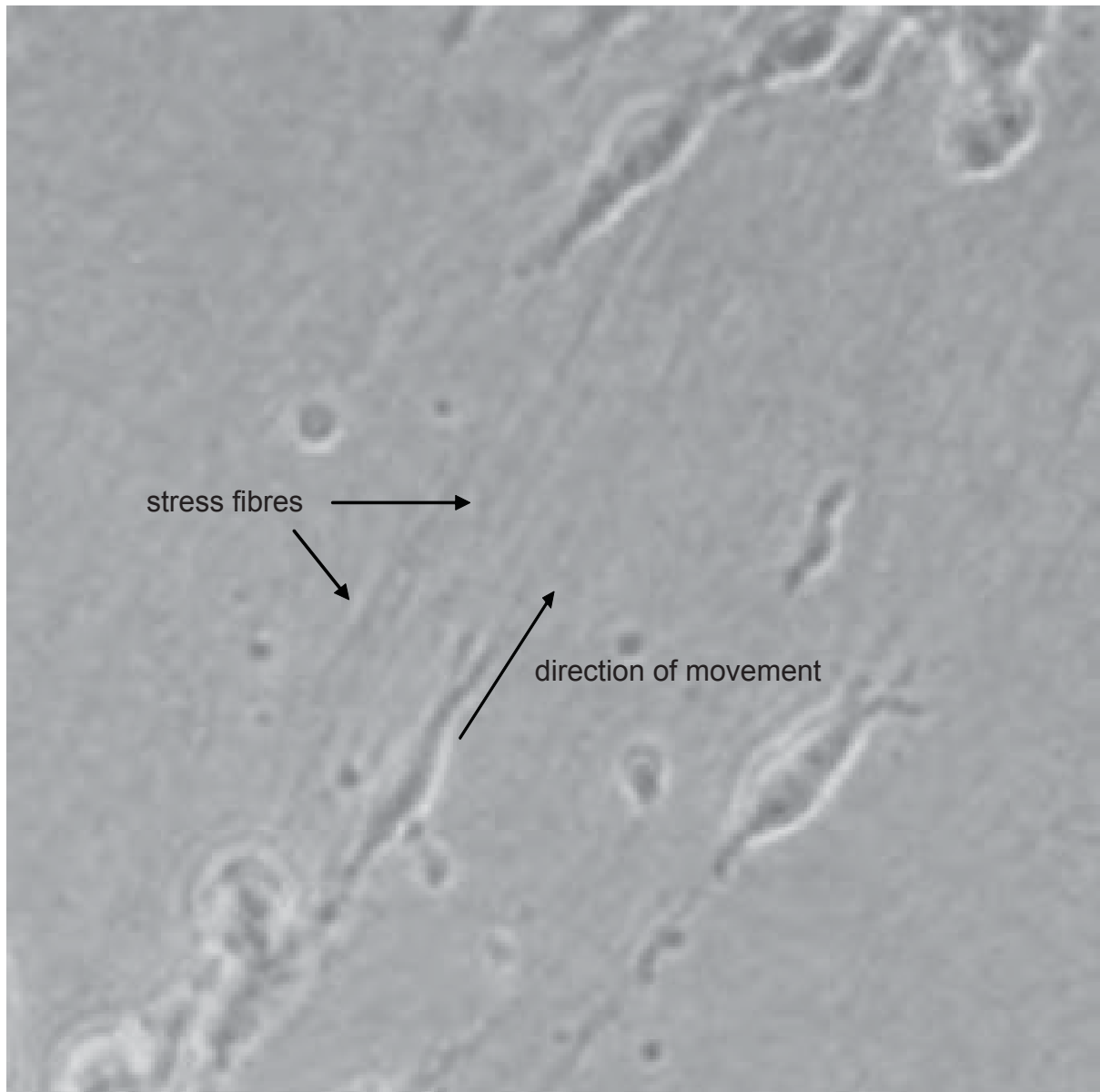


Figure 5 Elongated MMTV-neu mammary tumor cells align in the direction of stress fibres in type 1 collagen.

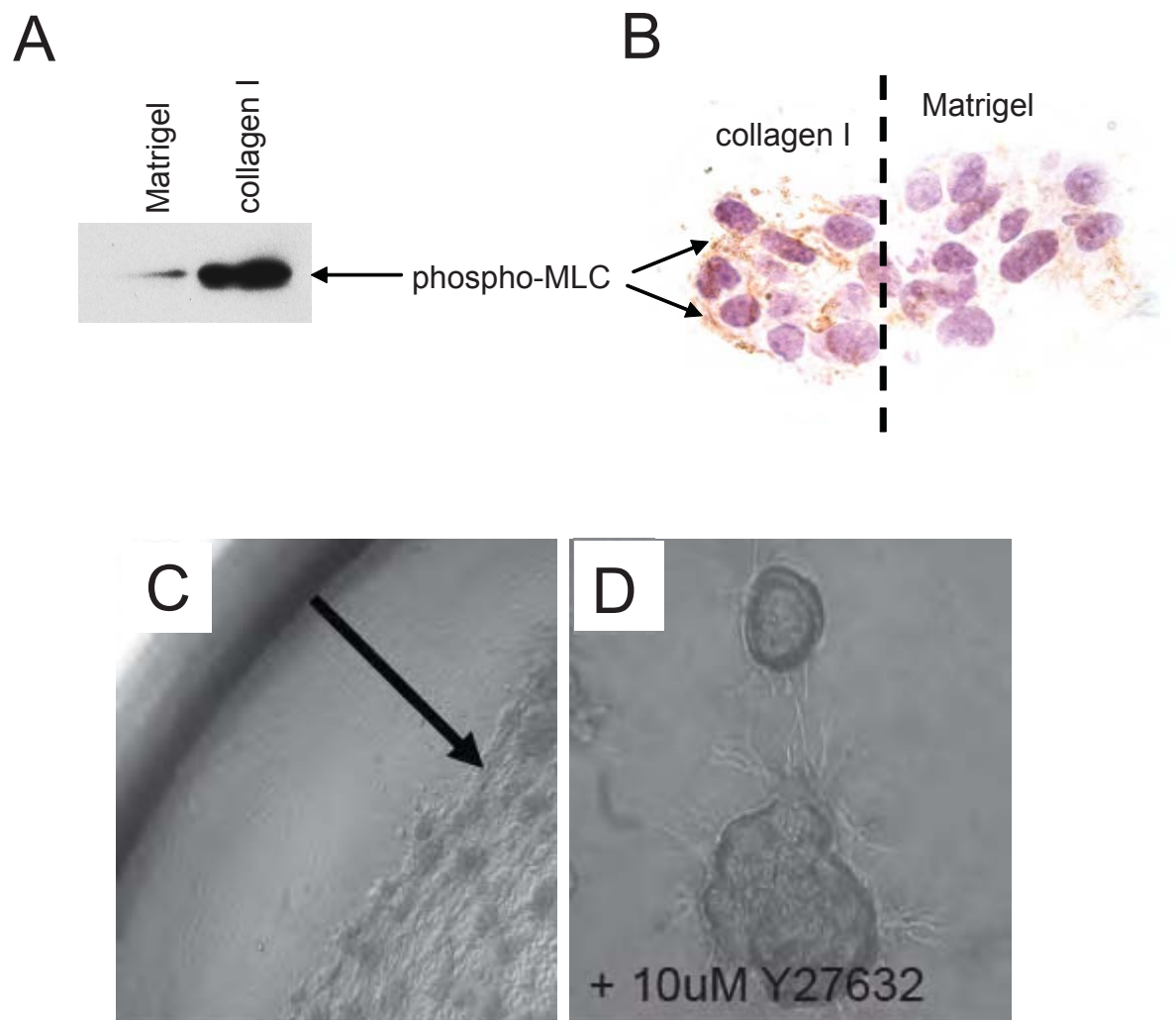


Figure 6

Collagen I induces MLC phosphorylation and actomyosin contractility. (A) Detection of MLC phosphorylation in collagen I by western blot and (B) immunohistochemistry of paraffin-embedded tumor cell acini. (C) Contraction of the collagen I gel away from the side of the cell culture dish. (D) Inhibition of collagen I contraction following addition of a Rho-kinase (ROCK) inhibitor (Y27632).

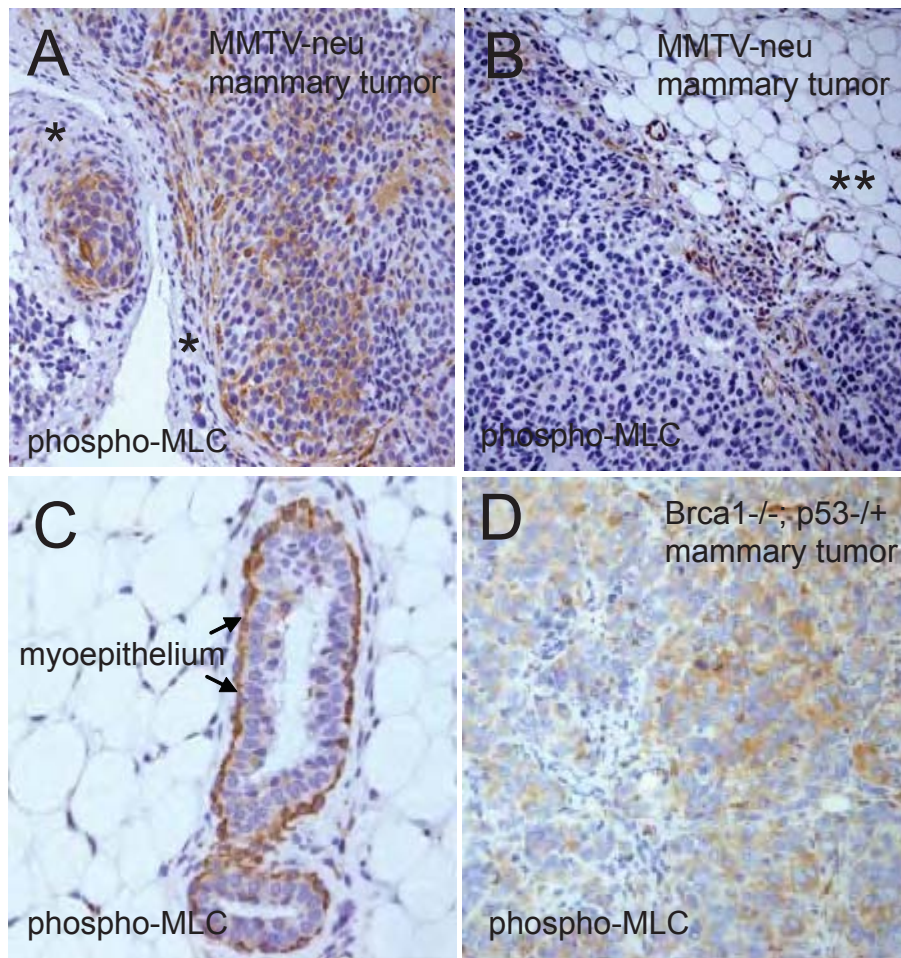


Figure 7

MMTV-neu mammary tumors acquire basal-like properties in a dense tumor stroma. (A) MLC phosphorylation (brown) adjacent to dense, fibrous stroma (*), compared to (B) absence of staining in pliable, fat-rich stroma (**). (C) Phospho-MLC is restricted to the myoepithelial/basal layer in the normal gland, and in (D) basal-like mouse mammary tumors in Brca1-/-; p53-/+ mice.

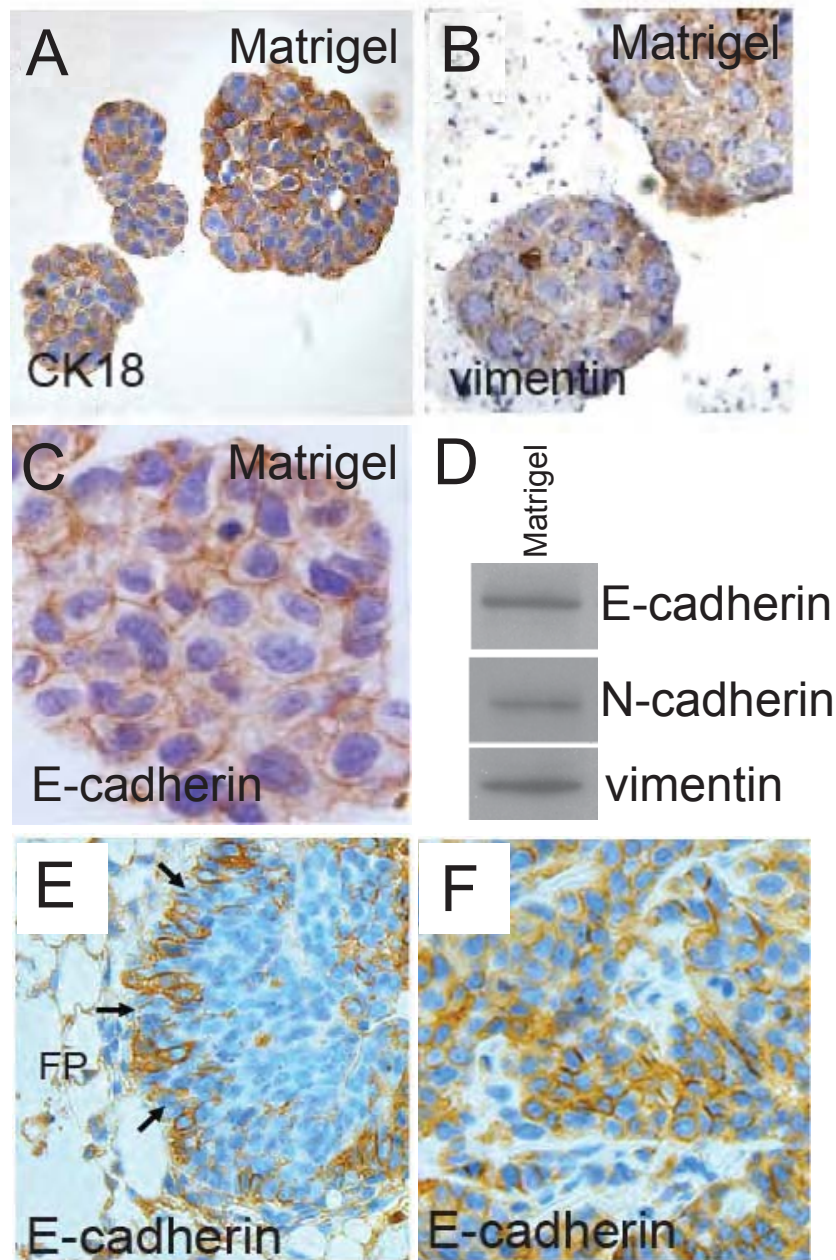


Figure 8

(A-D) MMTV-neu cells express EMT markers yet are non-invasive in Matrigel reconstituted basement membrane. (E) Cells undergoing EMT (E-cadherin-negative) in MMTV-neu mouse mammary tumors are non-invasive in a fatty stroma, compared to (F) E-cadherin-negative cells in a dense tumor stroma.