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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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 experimental results of year 1 primarily concern the characterization of tumor cell movement ex vivo. We have found that MMTV-neu and BRCA-1-/- mouse mammary tumor cells utilize both rounded (amoeboid) and elongated (mesenchymal) types of movement on type 1 collagen gels. The rounded and elongated types of movement were found to be dependent on Rho kinase (ROCK) and Rac1 activity, respectively. In addition, we have preliminary data suggesting that the elongated form of movement is driven by the fibrillar nature of type 1 collagen. This observation may have important implications for understanding the mechanisms behind local invasion in primary breast tumors. Green fluorescent protein (GFP) labelling of MMTV-neu tumor cells will facilitate the future analysis of tumor cell morphology and movement in vivo. The characterization of cell movement, and the elucidation of the mechanisms behind the different types of movement and invasion, may assist in the development of targeted therapies for blocking the metastatic spread of breast cancer.					
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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 (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<sup>-/-</sup> mouse models of mammary tumorigenesis (Guy et al., 1992; McCarthy et al., 2007). The movement of MMTV-neu and BRCA-1<sup>-/-</sup> 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<sup>-/-</sup> derived mouse mammary tumors also exhibit both contractile and mesenchymal behavior on a collagen gel (Figure 1B). Due to difficulty in maintaining BRCA-1<sup>-/-</sup> 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<sup>-/-</sup> 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.

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. The combination of cell biology and cell signalling 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

## 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 ROCKI and ROCKII 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 2 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 1 of the funded research program. In most cases, the experiments were performed according to the schedule outlined in the SOW of the original funding application. These results included confirmation that MMTV-neu and BRCA-1<sup>-/-</sup> mouse mammary tumor cells can adopt alternative modes of movement ex vivo (rounded and elongated). The experiments also generated preliminary observations that the rounded and elongated types of movement required Rho kinase (ROCK) and Rac1 activity, respectively. The results of these experiments, together with the successful GFP labelling of MMTV-neu tumor cells, provide a basis for experiments in years 2 and 3, which will examine the modes and mechanisms of mammary tumor cell invasion in vivo. In addition to the experiments proposed in the original SOW, we have evidence that mesenchymal tumor cell movement is driven by the composition of the extracellular matrix (ECM), specifically type 1 collagen. The combined experimental results from year 1, therefore, already offer important insights into the behavior of mammary tumor cells during migration and invasion.

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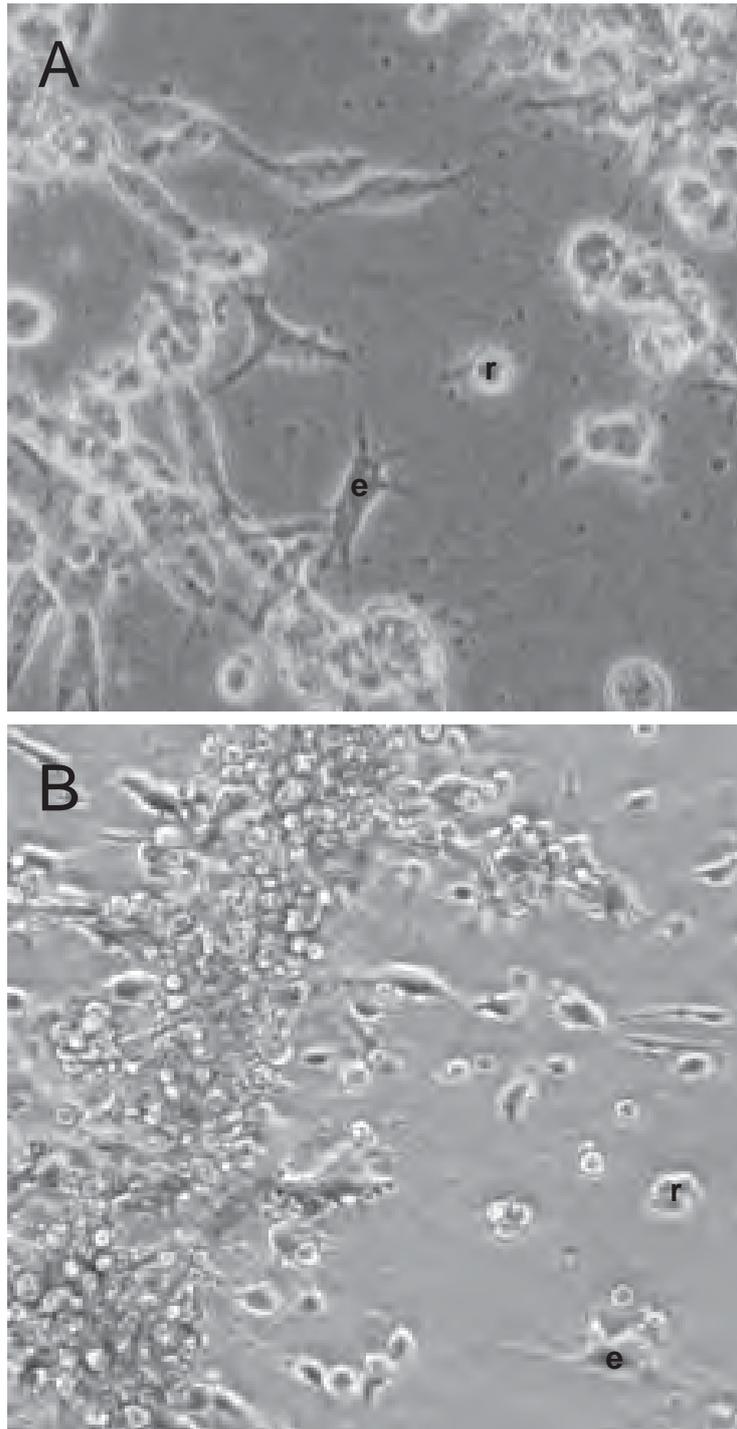
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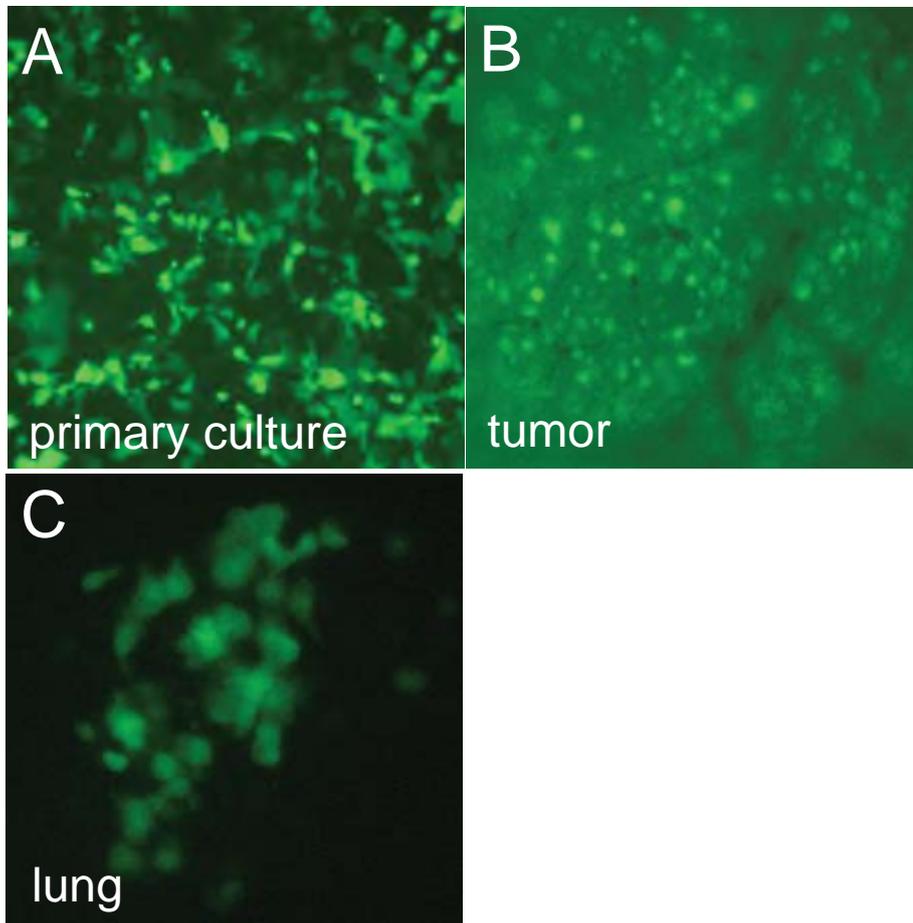
## APPENDICES



**Figure 1**

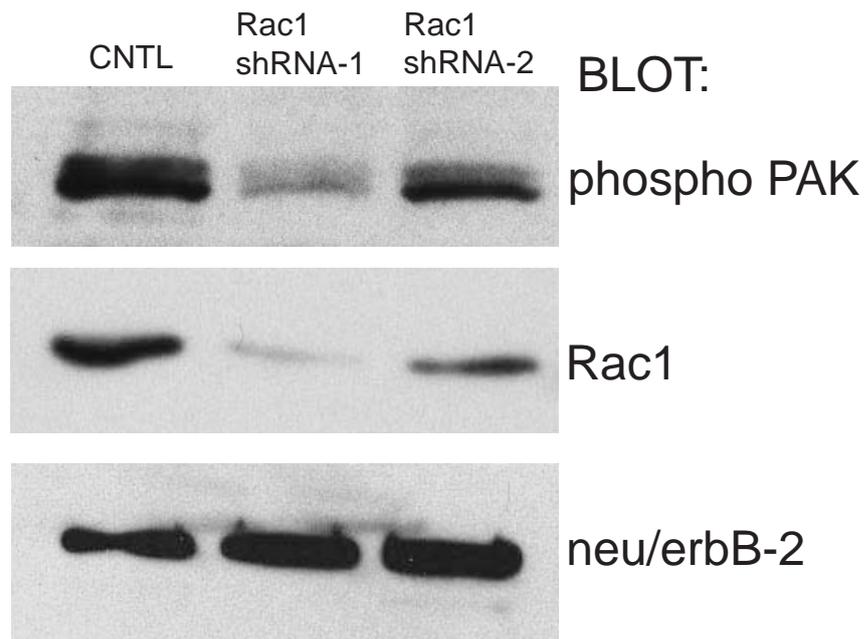
Primary mouse mammary tumor cells utilize both rounded and elongated forms of movement ex vivo. (A) Still image from time-lapse video microscopy of MMTV-neu-derived mouse mammary tumor cells, showing both rounded (r) and elongated (e) morphologies on a thick gel of type 1 collagen.

(B) The same analysis repeated for BRCA-1<sup>-/-</sup> mouse mammary tumor cells, again showing both rounded and elongated cells.



**Figure 2**

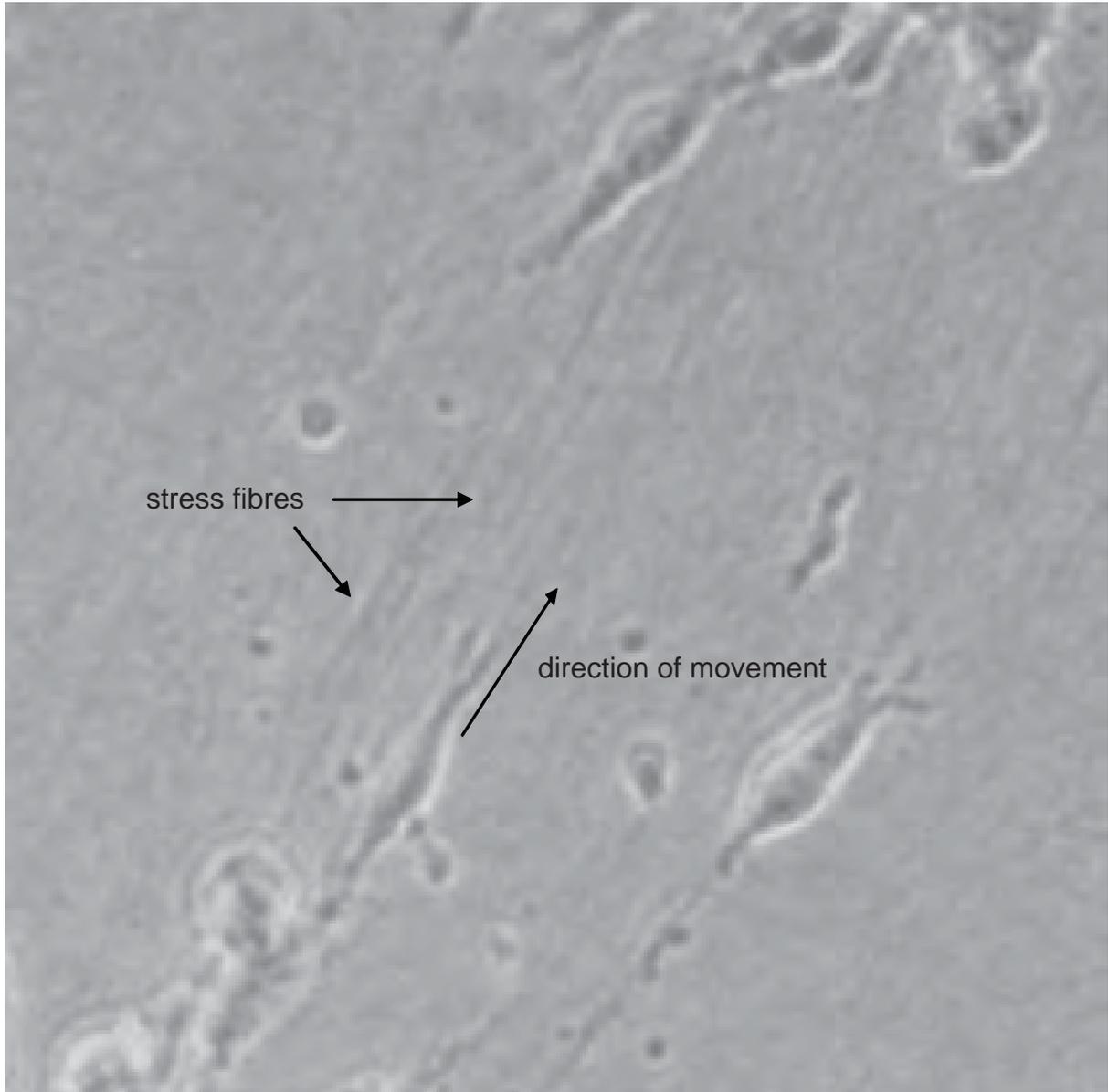
MMTV-neu-derived mouse mammary tumor cells expressing green fluorescent protein (GFP) can be imaged in vivo. (A) Monolayer culture of MMTV-neu cells stably expressing GFP. (B) Confocal image of mouse mammary tumor 3 weeks after transplantation of GFP-expressing MMTV-neu cells into mammary fat pad. (C) 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 5** Elongated MMTV-neu mammary tumor cells align in the direction of stress fibres in type 1 collagen.