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14. ABSTRACT The purpose of this funded program was to examine the regulation of cell shape and movement used by mammary tumor cells during local invasion and metastasis. We found that collagen-I, found in aggressive primary human breast tumors, promotes an invasive phenotype in a 3D culture system, through an increase in Rho/ROCK-mediated actomyosin contractility in HER2/neu-expressing mouse mammary tumor cells. High contractility is normally associated with the more aggressive basal-like breast cancers, providing evidence that collagen-I may be promoting a more aggressive phenotype in primary breast tumors. In addition, we have evidence that invasive properties of mammary tumors depends on the extracellular matrix environment, rather than expression of invasion-associated (mesenchymal) genes alone. Importantly, we have developed a novel 3D model of local invasion that occurs at the stromal interface of solid mammary tumors. This model was used to identify c-Src and ILK as critical mediators of EMT and local invasion, using small-molecule inhibitors. More importantly, we showed that changes in cell shape and 3D arrangement dramatically altered the sensitivity to ionizing radiation-induced death. This results have important clinical implications for the treatment of both primary and secondary tumors in metastatic breast cancer.					
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INTRODUCTION

The purpose of this funded research program was 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 FINAL 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 (collagen-I) (Figure 1A shows a still image taken from a time-lapse video of MMTV-neu cells on a thick bed of collagen-I). 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 nonetheless continued in year 2.

Task 1 included 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 was to be used for determining the modes of movement used by mouse mammary tumor cells during local invasion and metastasis in vivo, and 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, the intravital whole-animal imaging of metastatic dissemination (Task 1(d)) was moved from year 1 to years 2 and 3 of the program.

Several experiments were completed ahead of schedule in year 1. Year 2 of the program (Task 2), for example, 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 collagen-I, 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 continue 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 FINAL 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 3-D 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 year 2 of 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 Matrigel express markers of a luminal epithelial origin (E-cadherin and cytokeratin 18), as well as markers of the more invasive mesenchymal phenotype (Ncadherin, 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 compliant fatty stroma, as compared to the same cells in a more dense, stiff 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 summarizing the first 24 months of experimentation, our work remained 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.

BODY OF FINAL REPORT MONTHS 25-36

The third and final year of the programme involved further characterization and expansion of the in vitro analysis of cell shape reported in year 2, and the generation of results which may have significant clinical importance for the treatment of metastatic breast cancer. Specifically, the ex vivo model of EMT was used to investigate the therapeutic implications of collagen-I-driven local invasion of solid mammary tumors. As shown in Figure 9, MMTV-neu-derived tumor cells form solid, tumor-like structures when cultured on a thick bed of Matrigel (Figure 9, from White et al., manuscript in preparation). Phase contrast and histological examination of these structures reveals a reliable recapitulation of non-invasive early mammary lesions in MMTV-neu mice in situ. When we presented these tumor-like spheres with a thick layer of collagen-I, however, cells at the tumor-stroma interface started undergoing an EMT-like morphological change within two to three hours (Figure 10A-C), from White et al., manuscript in preparation). These changes induced by the presence of collagen-I were also accompanied by the loss of E-cadherin/catenin epithelial adhesion complexes from the cell membrane, a canonical hallmark of the EMT (Figure 10D, E). Importantly, these changes occurred in the absence of exogenously added growth factors and growth media, indicating that the EMT was driven exclusively by the addition of fibrillar collagen-I alone. After 24 hours, invasive cells had disseminated throughout the collagen-I gel, in a process resembling the phenomenon of local invasion (Figure 10F-H). This phenomenon is strikingly similar to the dissemination of tumor cells observed in collagen-rich regions of MMTV-neu

mouse mammary tumors in situ (Figure 10I, from White et al., manuscript in preparation).

The recapitulation of local mammary tumor invasion ex vivo provided an experimental model to investigate potential anti-metastatic drugs, as well as the impact of cell shape on the biological properties of cells. Using this model to screen a small-molecule inhibitor library, we identified the c-Src kinase inhibitor Dasatinib as a potent drug for blocking EMT and local invasion in collagen-I (Figure 11A-F, from White et al., manuscript in preparation). The c-Src kinase has attracted considerable attention for its possible role in tumor cell migration and invasion, and for the reports that c-Src kinase activity is elevated in a majority of human cancers and mouse mammary tumors. Here we show that Dasatinib, which is currently in use in clinical trials, has a specific effect in blocking EMT and local invasion. We reported no impact on cell viability in this assay (data not shown). The role of c-Src as a downstream effector of integrin collagen receptors is reflected by the observation that local invasion was also blocked through the application of an integrin-blocking inhibitory antibody (Figure 11G-J).

This ex vivo model of local invasion was also used to investigate the role of another integrin effector, the integrin linked kinase or ILK, in metastatic disease. In this case, I was co-first author on a publication showing that the genetic ablation of ILK resulted in reduced metastatic properties of erbB2/neu-driven mouse mammary tumors in vivo (Pontier et al., 2010--Appendix). The ex vivo model developed in this funded program was used in this publication to show that the selective inhibition of ILK resulted in preferential death of cells undergoing local invasion in the pro-invasive collagen-I matrix. This observation provided a possible explanation for the reduced metastatic capacity of the ILK-null tumors, and identified ILK as a potential therapeutic target for metastatic breast cancer.

Cell morphology has been shown to be important for regulating various aspects of cell biology, including proliferation, survival, and the lineage commitment of progenitor or stem cells. Since metastatic cells will encounter an evolving stromal microenvironment, from primary tumor to distant target tissues, we wanted to use this model to investigate the biological impact of stroma-induced cell shape changes on drug resistance and the resistance to ionizing radiation. Interestingly, the death of invasive cells following ILK inhibition was consistent with observations of impaired survival pathways in the invasive cells, suggesting an inherent sensitivity to cell death during the invasion process (data not shown). To determine if these results had practical clinical implications, we subjected the assay to ionizing radiation at doses comparable to those used in the clinic. Consistent with the impaired survival signaling pathways in the invasive cells, we found that one dose of 5Gy radiation was effective in inducing caspase-mediated apoptosis of the invasive, disseminated cell population. Cells which were maintained in the solid, tumor-like arrangement showed, in contrast, no evidence of cell death for up to several days following radiation treatment. This phenomenon is now being investigated further, to identify pathways responsible for the differential response to radiation, and to screen for small-molecule inhibitors which may sensitize the more resistant cells. Since the polarity, morphology and 3-D arrangement of tumor cells is a dynamic process during disease progression, these results will help us understand how the stroma will influence radiation

and drug resistance through an impact on cell shape. These results have implications beyond the treatment of primary breast cancers. Since macroscopic metastatic lesions are re-established from disseminated cells during tumour cell growth in secondary sites, colonizing cells may have a survival advantage, even in the presence of adjuvant chemotherapy or radiation. Knowledge gained from the analysis of this 3-D model system, therefore, may have important implications for the treatment of both primary and secondary metastatic tumours.

The work resulting from year 3 of the funded program is currently being prepared as a manuscript for submission. Figures from the draft manuscript are presented in the Appendix (Figures 9-12).

With regards to personal training landmarks, I have acquired important new skills and knowledge during the three years of this funded research 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. In this regard, the three years of funded research has led to an opportunity to establish an independent group within the Breakthrough Breast Cancer Centre in London, UK. I am now independently pursuing questions concerning the role of cell morphology in metastatic breast cancer, using the models and knowledge gained from the Department of Defense-funded program.

KEY RESEACH 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 RESEACH 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

KEY RESEACH ACCOMPLISHMENTS (year 3)

- showed that recapitulated solid mammary tumors undergo local invasion when presented with collagen-I ex vivo
- showed that EMT and local invasion can be selectively blocked using a small-molecule inhibitor of the c-Src kinase
- reported that invasive cells are selectively killed by a small-molecule inhibitor of the integrin linked kinase (ILK) (Published)
- reported that the inhibition of invasion through targeting ILK is associated with reduced metastasis in the MMTV-neu mouse mammary tumor model
- showed that cells undergoing invasion are more sensitive to radiation-induced cell death, compared to cells maintained in a solid, tumor-like arrangement
- currently preparing manuscript regarding the role of collagen-I in driving cell shape and invasion, with figures provided in the Appendix (Figures 9-12)

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.

Abstract: Institute of Cancer Research Centenary Conference, June 8-10, 2009:

During metastatic disease progression, tumor cells face a constantly changing microenvironment, both within the primary tumor and in secondary target tissues. In addition to stromal cells, inflammatory cytokines and growth factors, the tumor microenvironment consists of structural proteins of the basement membrane and extracellular tissue matrix (ECM). Unlike the cellular and growth factor/cytokine components, however, little is known about the role of ECM proteins in promoting metastatic disease progression. One of the fibrillar ECM proteins, collagen-I, has been found to accumulate in late-stage breast tumors, and increased collagen-I biosynthesis can be detected in patients undergoing aggressive disease relapse. Here we have developed a 3D assay to investigate the impact of collagen-I on solid mammary tumor growth and architecture *ex vivo*. When grown on a thick bed of Matrigel™ reconstituted basement membrane, HER2/neu-expressing mouse mammary tumor cells recapitulate features of solid mammary tumors found *in vivo*. In the presence of collagen-I, however, cells at the tumor margin rapidly undergo changes in shape and invasive behavior resembling the epithelial-mesenchymal transition (EMT), including reorganization of cortical actin, disassembly of E-cadherin/catenin adhesion complexes, and the acquisition of a highly invasive phenotype. These changes result in disruption of the tumor margin, resulting in a phenomenon resembling local tissue invasion. Interestingly, cells in the solid tumor-like arrangement express both epithelial and mesenchymal markers, yet only undergo morphological EMT in the presence of collagen-I. We have also shown that the invasive properties induced by collagen-I require c-Src kinase activity and its downstream effector FAK. Inhibition of c-Src activity restores the tumor margins, and acts as a switch between epithelial and mesenchymal morphologies both in 2D and 3D. Together, our results indicate that collagen-I can promote local invasion in primary breast tumors by driving a more mesenchymal cellular phenotype, and that this EMT-like switch can be reversed through inhibition of the c-Src kinase.

Publication: Integrin linked kinase has a critical role in erbB2 mammary tumor progression: implications for human breast cancer. Pontier SM*, Huck L*, White DE*, Rayment J, Sanguin-Gendreau V, Hennessy B, Zuo D, St-Arnaud R, Mills GB, Dedhar S, Marshall CJ, Muller WJ. *Oncogene*. 2010 Jun 10;29(23):3374-85. Epub 2010 Mar 22. *co-first authors. (see Appendix).

CONCLUSION

Several important experimental observations were reported at the end of 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.

Most of the Tasks involving in vitro analysis of tumor cell movement outlined in the SOW were accomplished within the first two years of this research program. The biochemical and histological analysis of the proposed mouse mammary tumor models, however, did not reveal reciprocal expression and regulation between Rho/ROCK and Rac1 signaling, as was hypothesized based on other cell lines and cell types. These tumor types, therefore, did not represent appropriate models for understanding the reciprocal regulation of these pathways in driving metastasis in vivo. In addition, we found that EMT-like invasion in vitro, unexpectedly, did not require the activity of matrix metalloproteinases (MMPs), which was a proposed experiment in Task 3a (data not shown).

Year 3, however, saw important developments with regards to the ex vivo modelling and analysis of stromal influence on cell shape and invasive movement. The ex vivo model of local invasion in solid tumors was utilized in a publication involving the role of ILK in mammary tumor metastasis (Pontier et al., 2010). In addition, the identification of Dasatinib as an inhibitor of EMT and local invasion demonstrates the utility of this assay for the large scale screen of small-molecule inhibitors which can block the transition to a more aggressive, metastatic tumor cell type. More importantly, we show that the regulation of cell shape and 3D arrangement of tumor cells has a dramatic impact on their biological properties. In this case, we found that the acquisition of an elongated, invasive phenotype in single, disseminated cells resulted in sensitization to radiation-induced cell death. The changes in cell shape in this assay were due entirely to addition of collagen-I. Since tumor cells encounter a constantly evolving stromal microenvironment during growth and dissemination, understanding the impact of the stroma on cell shape and biological outcome will have important consequences for the clinical management of metastatic disease.

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APPENDICES

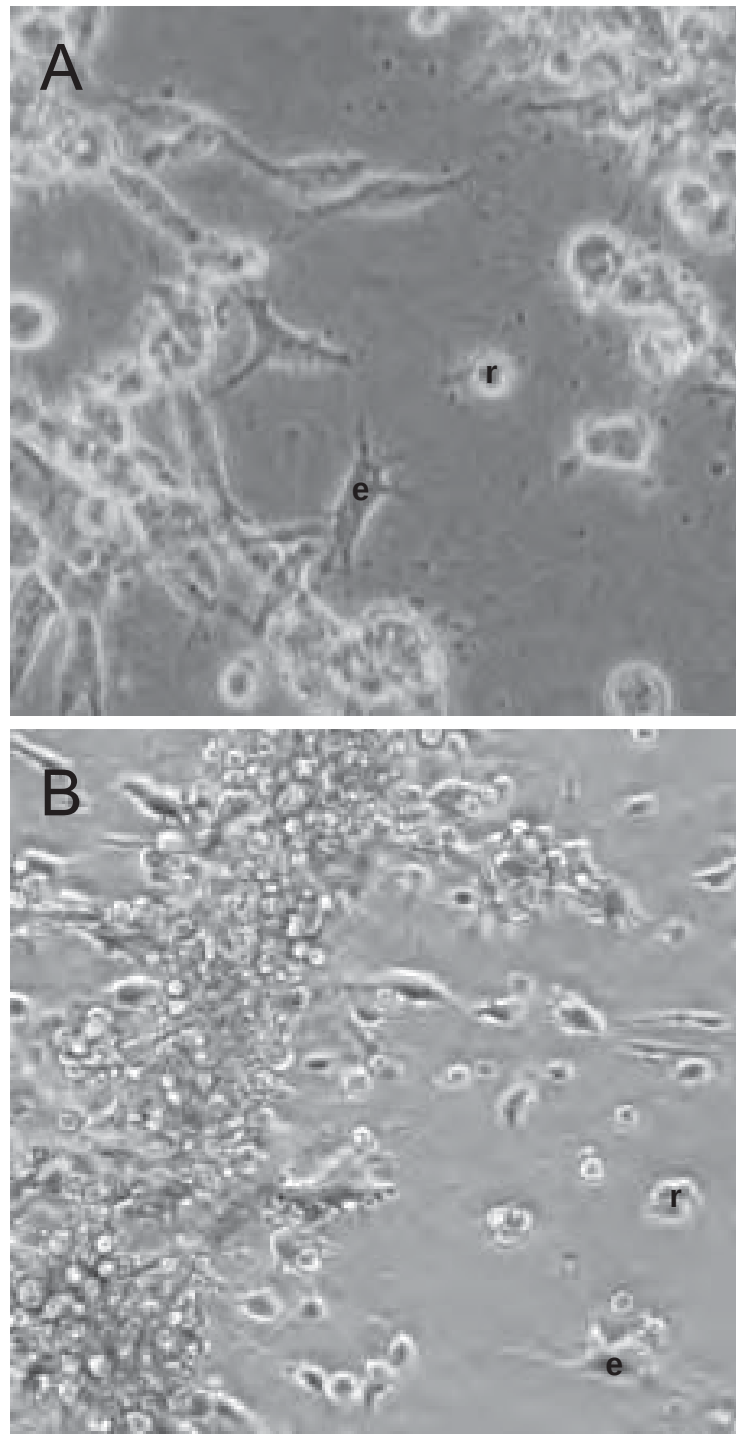


Figure 1

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.

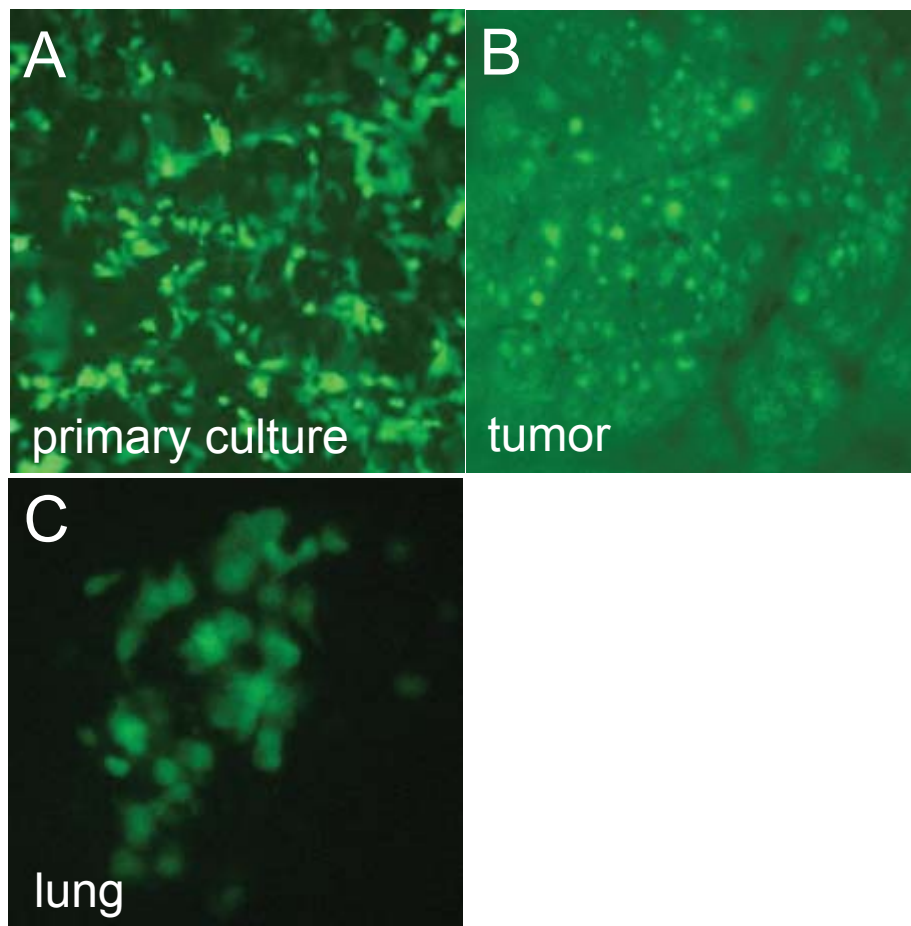


Figure 2

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.

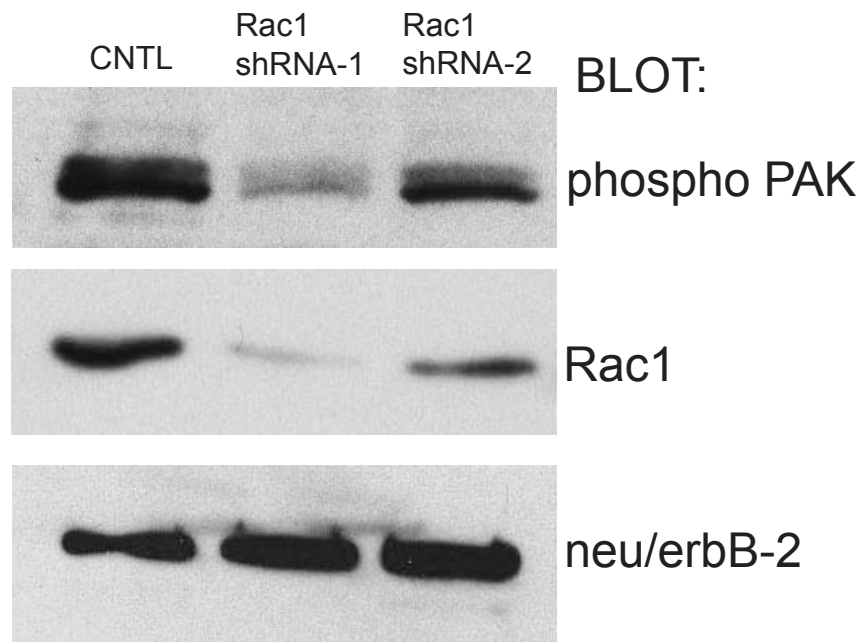


Figure 3

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).

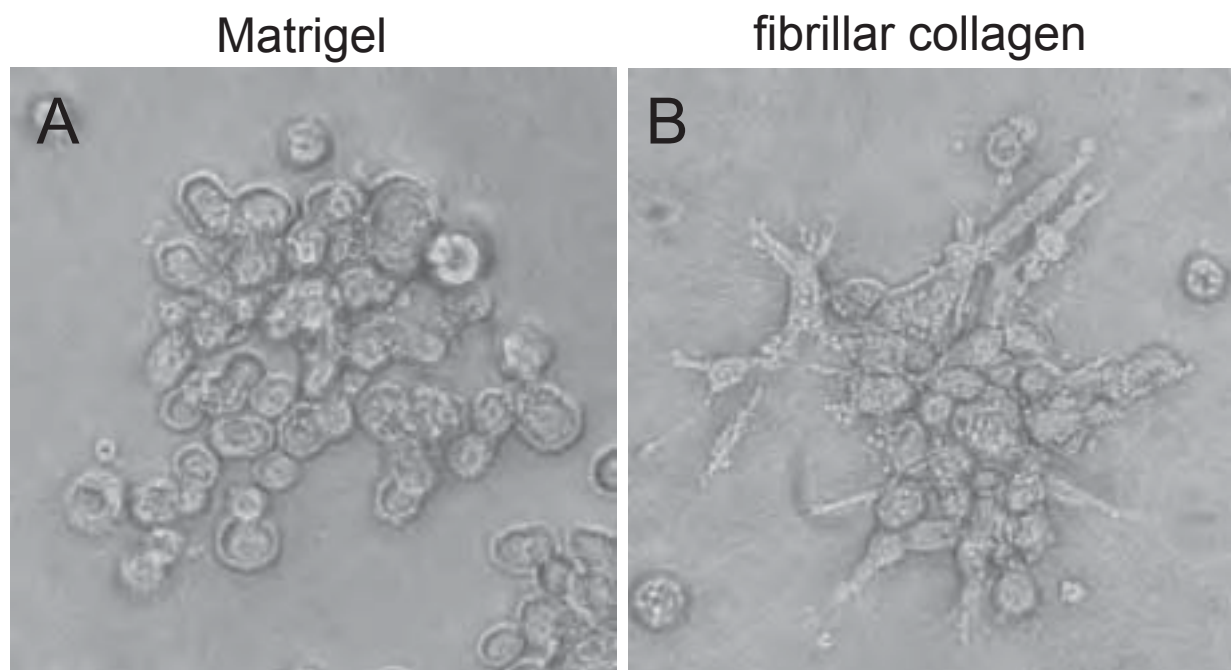


Figure 4

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.

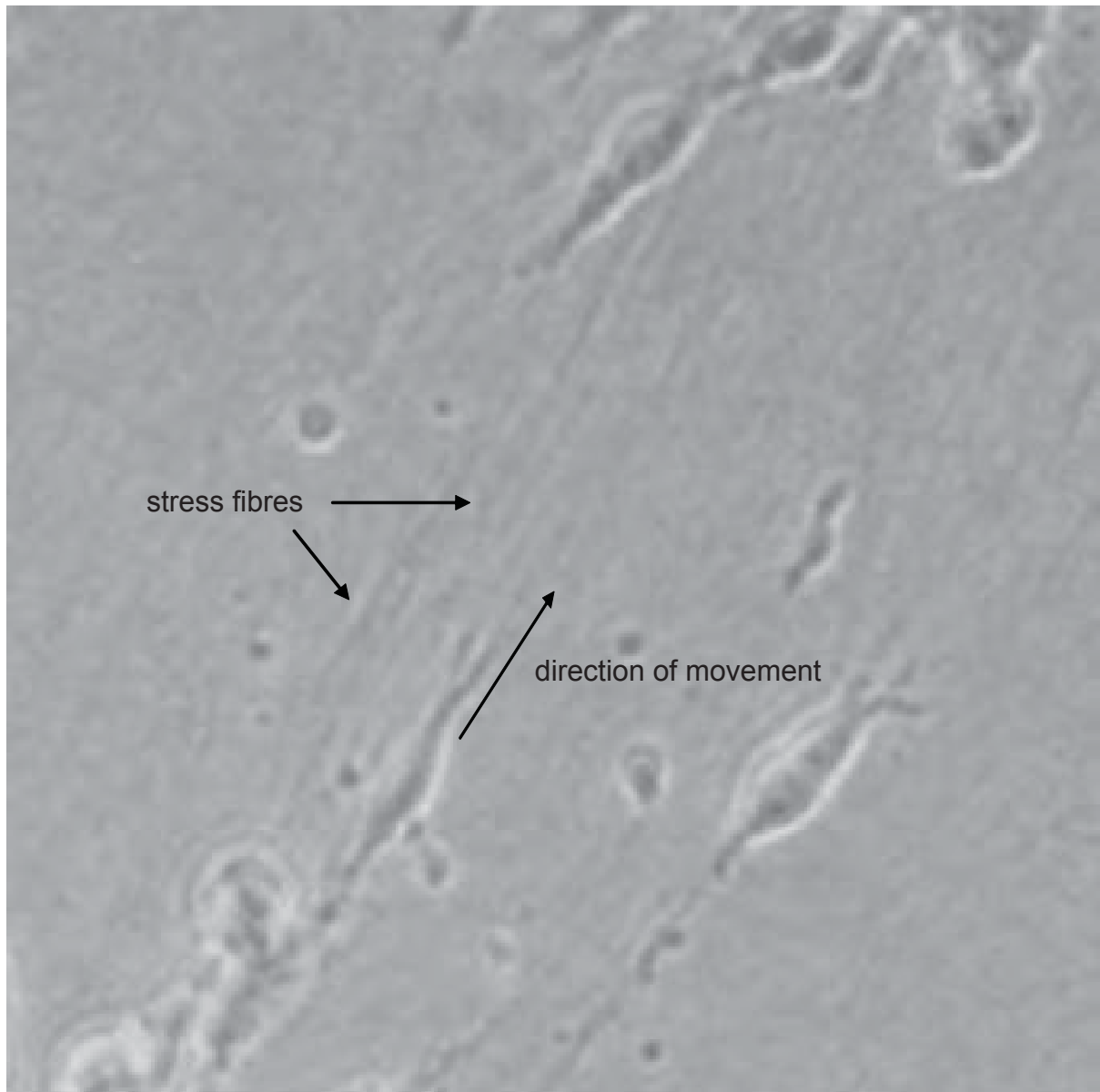


Figure 5 Elongated MMTV-neu mammary tumor cells align in the direction of stress fibres in type 1 collagen.

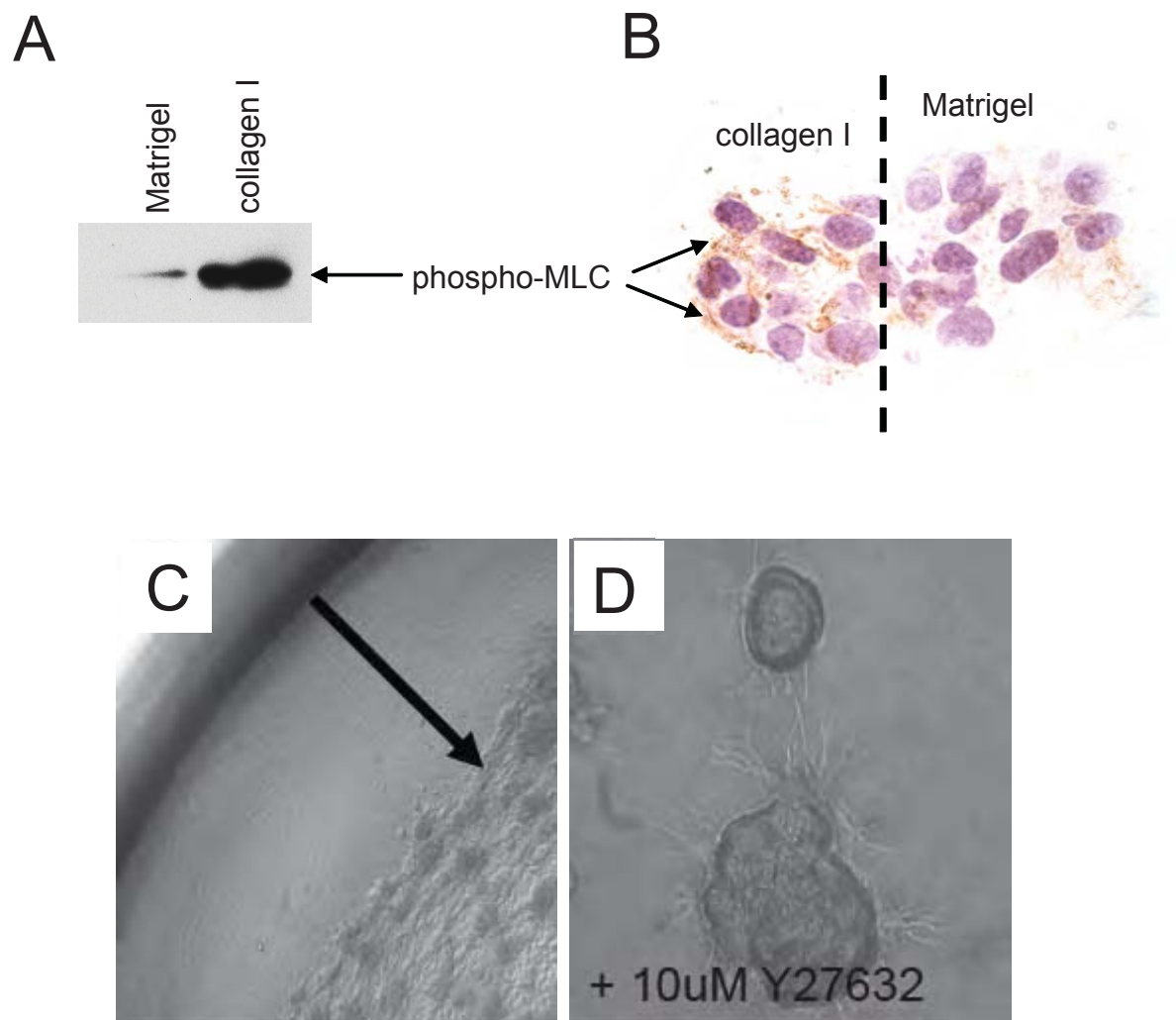


Figure 6

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).

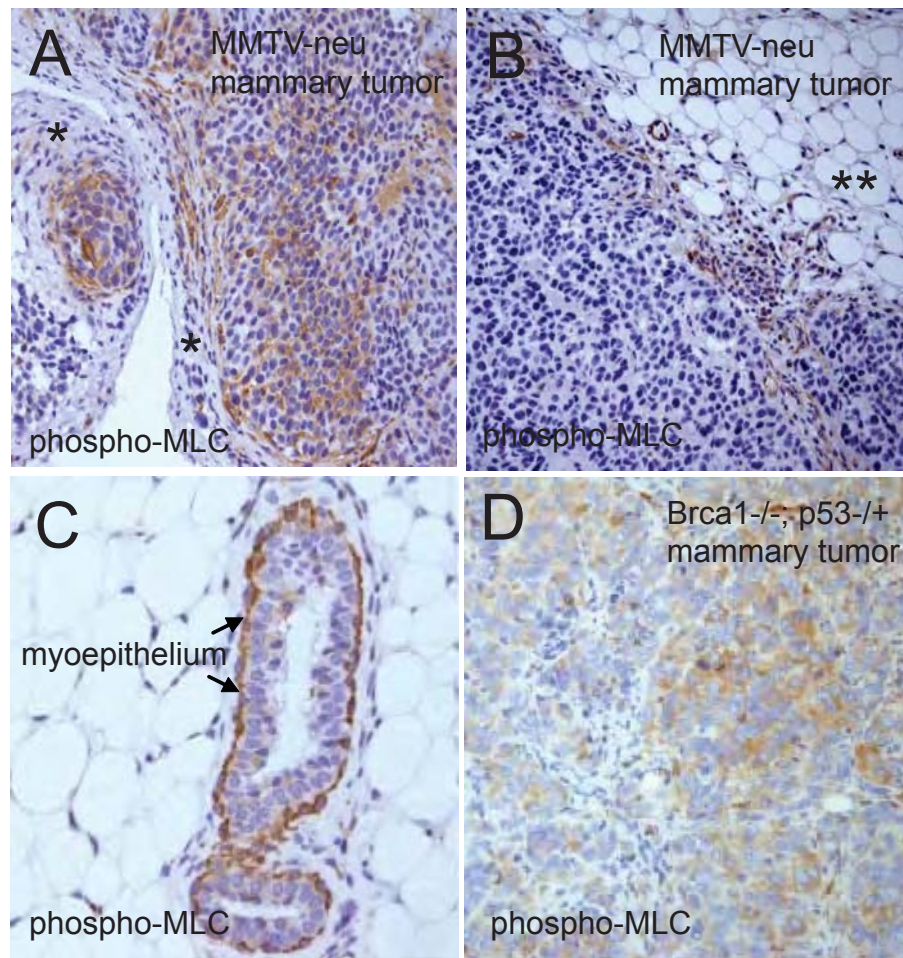


Figure 7

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.

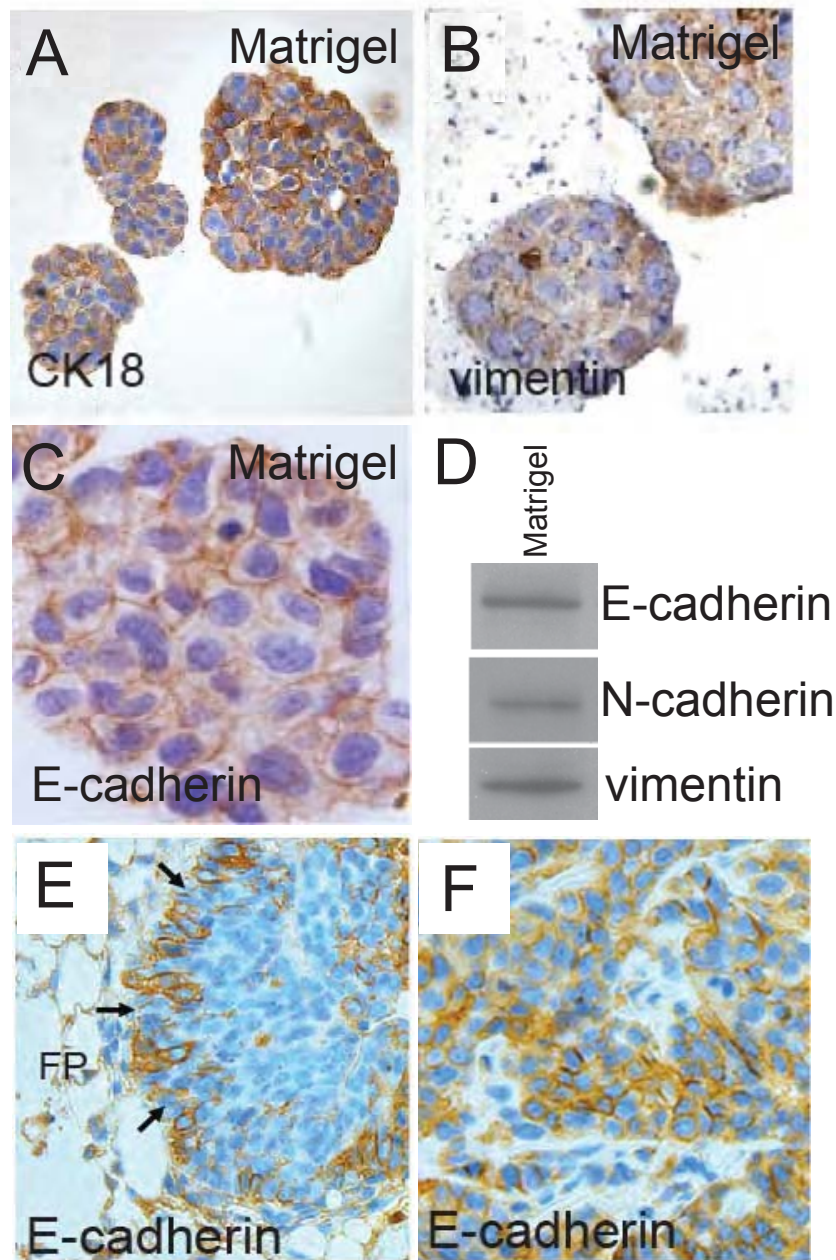


Figure 8

(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.

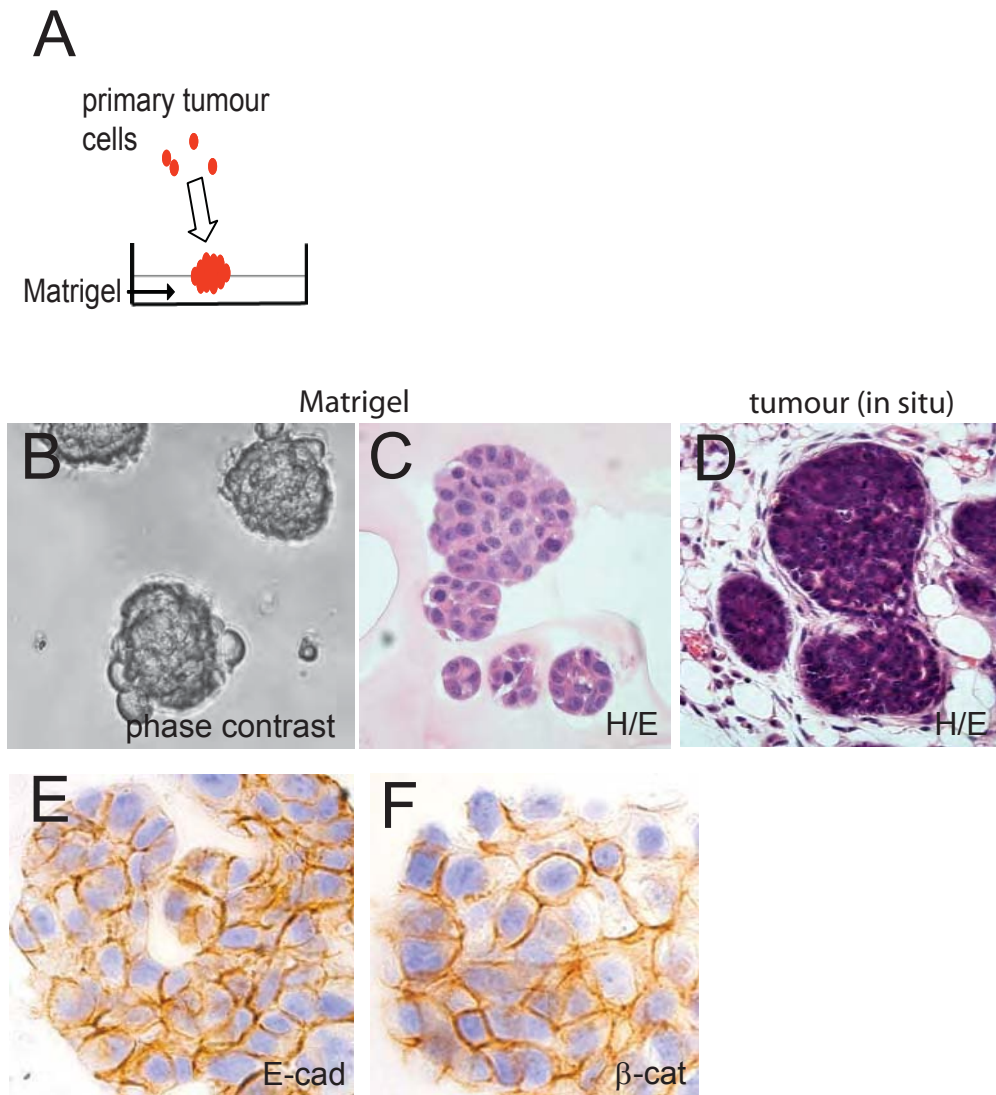


Figure 9

MMTV-neu-derived tumour cells recapitulate solid mammary tumors on Matrigel. (A) Experimental setup. (B) Phase contrast and (C) H&E stained section of tumor-like spheres. (D) H&E stained section of MMTV-neu mouse mammary gland, showing non-invasive tumor embedded in mammary fat pad. (E, F) Immunostaining for E-cadherin and β -catenin, respectively. Figure is from White et al., manuscript in preparation.

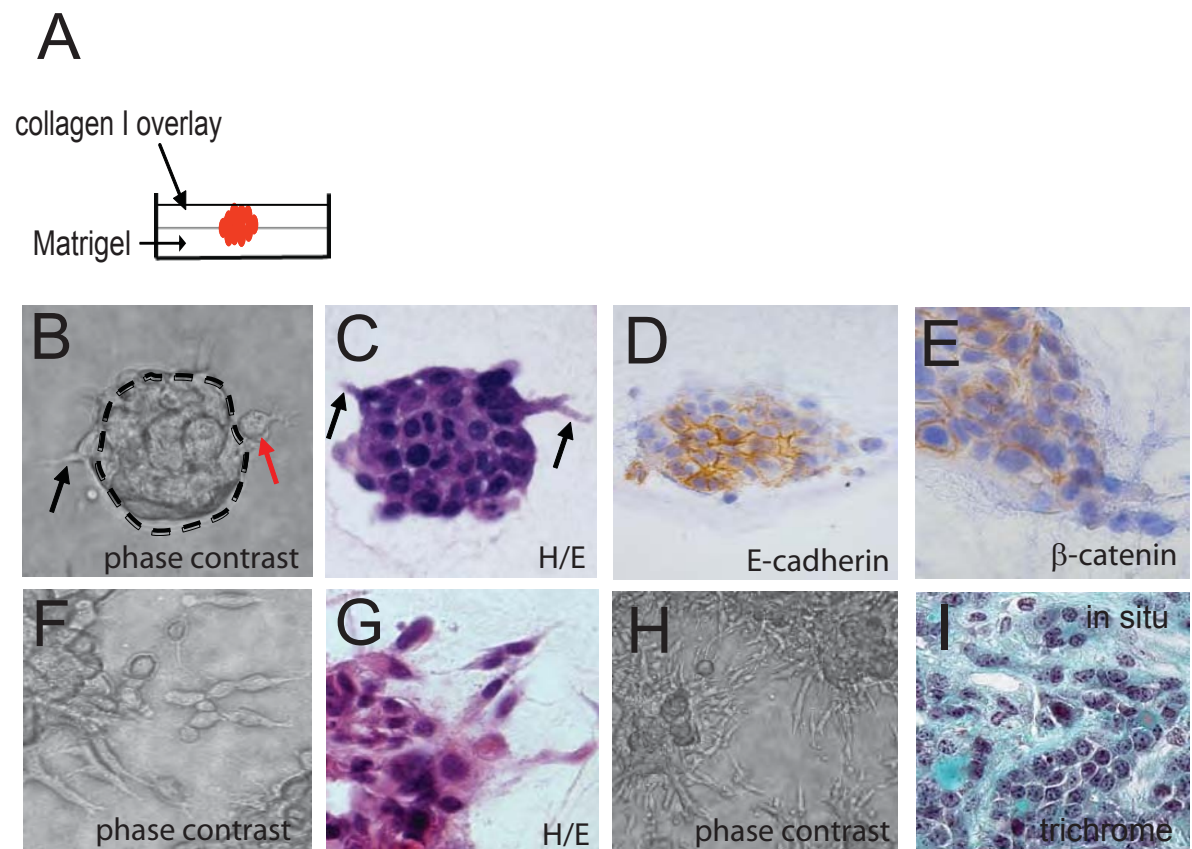


Figure 10

Collagen-I induces EMT and local invasion of tumor-like spheres in 3D. (A) Experimental setup. (B) Phase contrast and (C) H&E stained section of tumor cells undergoing elongation and invasion in collagen-I. (D,E) Immunostaining for E-cadherin and β -catenin loss at the collagen-I interface, respectively. (F-H) Invasion and dissemination into the collagen-I gel after 24 hours. (I) Trichrome staining of tumor section from MMTV-neu mouse, showing disseminated cells (dark blue) in collagen-rich regions (light blue). Figure is from White et al., manuscript in preparation.

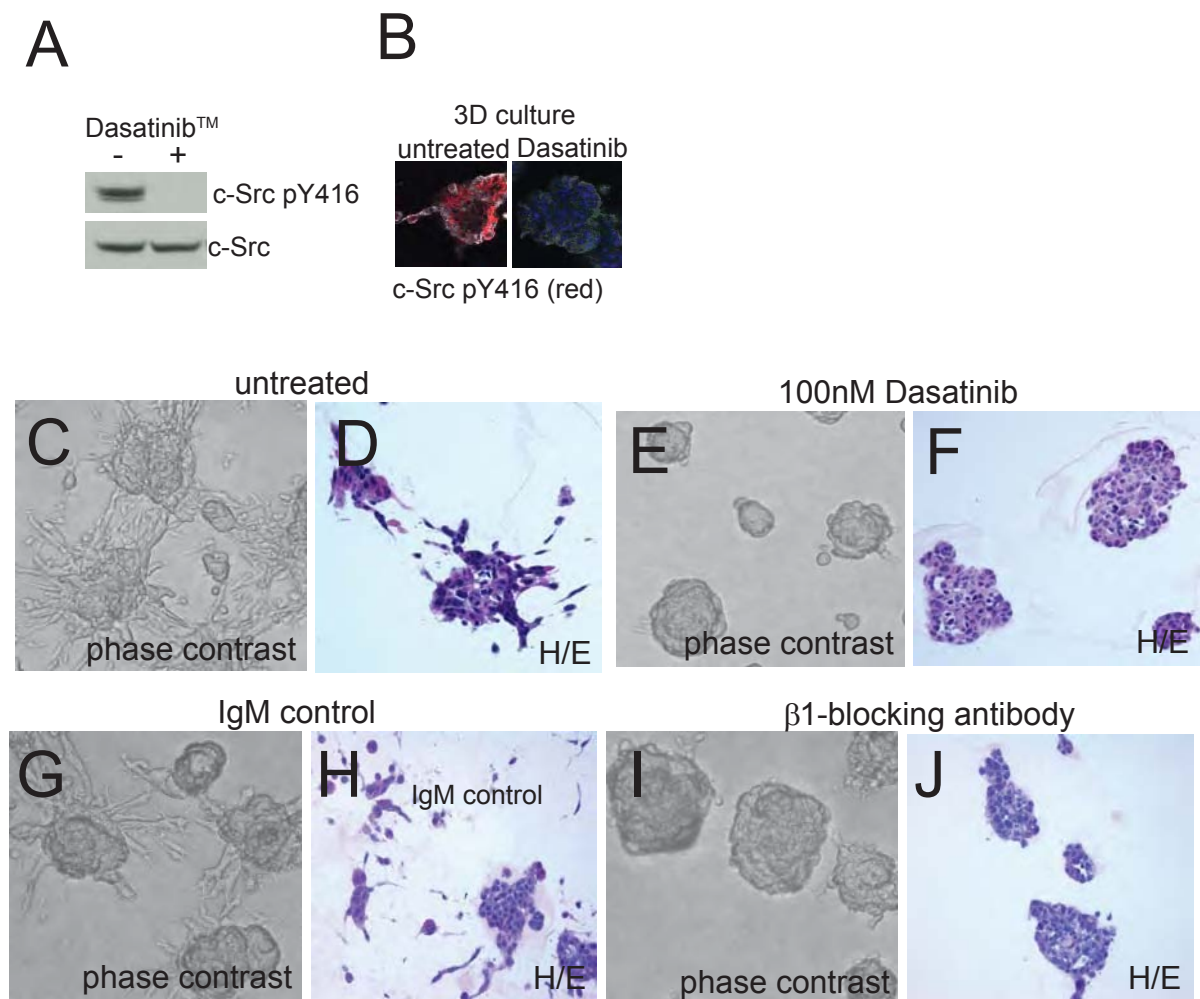


Figure 11

Invasion in collagen-I requires c-Src kinase activity and β 1-integrin binding. (A) Western blot and (B) immunofluorescence showing inhibition of c-Src kinase activity using Dasatinib. (C,D) Untreated tumor-like spheres undergoing local invasion in collagen-I. (E,F) Inhibition of local invasion following treatment with Dasatinib. Spheres were also incubated with (G,H) control IgM antibody and (I,J) β 1-integrin blocking antibody (Ha2/5; BD Biosciences) prior to addition of collagen-I. Figure is from White et al., manuscript in preparation.

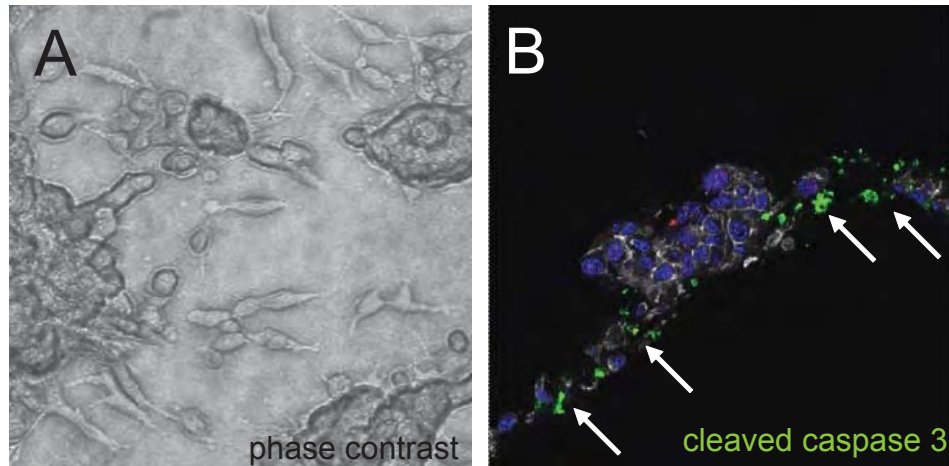


Figure 12

The extracellular matrix dictates response to radiation through an impact on cell shape and 3-D orientation. (A) Solid mammary tumor cells undergoing local invasion in collagen-I. (B) Immunofluorescence analysis of collagen-I culture following 5Gy ionizing radiation. Note that cell death is limited exclusively to invasive cells (cleaved caspase 3, green staining, white arrows). DAPI appears blue while actin is in grey. Figure is from White et al., manuscript in preparation.

ORIGINAL ARTICLE

Integrin-linked kinase has a critical role in ErbB2 mammary tumor progression: implications for human breast cancer

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Elevated expression of the integrin-linked kinase (ILK) has been observed in a variety of cancers and has been further correlated with poor clinical outcome. Here, we show that mammary epithelial disruption of ILK results in a profound block in mammary tumor induction. Consistent with these observations, inhibition of ILK function in ErbB2-expressing cells with small molecule inhibitor or RNA interference resulted in profound block in their *in vitro* invasive properties due to the induction of apoptotic cell death. The rare ILK-deficient tumors that eventually arose overcame this block in tumor induction by an upregulation of ErbB3 phosphorylation. These observations provide direct evidence that ILK has a critical role in the initiation phase of ErbB2 tumor induction.

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Keywords: integrin-linked kinase; ErbB2; mammary tumorigenesis

Introduction

The integrin-linked kinase (ILK) is a modular protein with separate scaffolding and kinase-like domains that mediates the physical and functional coupling of integrins to growth factor signaling (Hannigan *et al.*, 2005; McDonald *et al.*, 2008a). ILK modulates integrin-mediated adhesion by directly interacting with the cytoplasmic tails of $\beta 1$ - or $\beta 3$ -integrin receptors (Hannigan *et al.*, 1996), and complexes with Parvin A/B (Attwell *et al.*, 2003; Mongroo *et al.*, 2004) and PINCH (Tu *et al.*, 1999). More recently, ILK has also been shown to have an instrumental role in the proper organization of the mitotic spindle during cell division (Fielding *et al.*, 2008). In addition to these critical scaffold functions, ILK can modulate a number of cell

survival and proliferation signaling pathways, including the AKT/GSK3 pathway and mTOR signaling. ILK is involved in the phosphorylation of two serine/threonine kinases, GSK3 β and Akt1 (Delcommenne *et al.*, 1998). ILK-mediated phosphorylation of GSK3 results in GSK3 β auto-inhibition, and the consequent stabilization of β -catenin and cyclin D1 (Radeva *et al.*, 1997; D'Amico *et al.*, 2000). Whereas ILK-induced AKT phosphorylation on its serine residue S473 increases cell survival by promoting anoikis resistance (Attwell *et al.*, 2000), phosphorylation of this residue also induces VEGF-dependent angiogenesis in cancer cell lines (Tan *et al.*, 2004). Conversely, inhibition of ILK or ILK-PINCH function markedly decreases AKT phosphorylation in number of normal and cancer cell lines (Persad *et al.*, 2000; Fukuda *et al.*, 2003a, 2003b; Hannigan *et al.*, 2005; Koul *et al.*, 2005; Younes *et al.*, 2005; Troussard *et al.*, 2006). Recent data have also shown that ILK functionally interacts with mTORC2 (Troussard *et al.*, 2006), through a direct interaction with Rictor, a component of the mTOR complex 2 (McDonald *et al.*, 2008b). Taken together, these observations suggest that ILK may mediate its effects through multiple distinct molecular pathways.

Direct evidence of the importance of ILK in mammary tumor progression derives from studies with transgenic mice. Mammary epithelial expression of ILK in transgenic mice using the MMTV promoter is associated with the induction of mammary epithelial hyperplasias that rarely progress to full malignancy (White *et al.*, 2001). Interestingly, the tumors that eventually occur in these strains exhibit hallmarks of epithelial to mesenchymal transition (EMT) and no longer productively express the transgene but express elevated levels of the endogenous ILK gene (White *et al.*, 2001).

Whereas these studies implicate elevated ILK expression in mammary tumor progression, its role in cancers induced by activated growth factor receptors such as ErbB2 is unclear. To investigate the role of ILK in ErbB2 tumor progression, we have assessed the effect of the targeted disruption of ILK in mammary epithelium on both normal mammary development and ErbB2 tumor progression. To accomplish this, we have interbred mice bearing a conditional ILK allele (Terpstra *et al.*, 2003) to

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separate transgenic mice co-expressing ErbB2 and Cre recombinase in the mammary epithelium (Ursini-Siegel *et al.*, 2008). Although mammary epithelial disruption of ILK had little impact on normal mammary gland development (Supplementary Figure S1A), loss of ILK function in ErbB2-expressing epithelium resulted in a profound proliferative block that was correlated with a dramatic impact in ErbB2 tumor onset and penetrance. However, the rare ILK-deficient tumors that eventually arose regained their capacity to proliferate that was correlated with dramatic upregulation of ErbB3 tyrosine phosphorylation. Finally, using an *in vitro* dissemination assay, we show that abrogation of ILK function in established ErbB2 mammary tumor cells results in block in invasion that was further correlated with induction of apoptotic cell death. Collectively, these observations suggest that ILK has a critical role in ErbB2 mammary tumor progression.

Results

Targeted disruption of ILK in mammary epithelium expressing activated ErbB2 results in a dramatic delay in mammary tumor development

To determine the effects of ILK deletion on normal mammary gland development, we interbred mice carrying the conditional ILK alleles to a separate strain of mice expressing the Cre recombinase under the control of the MMTV promoter (Figure 1a). To confirm the loss of ILK expression in the mammary epithelium of mice bearing the conditional ILK allele, we performed immunofluorescence with ILK-, Cre- and Keratin 8-specific antibodies on paraffin-embedded sections of mammary glands derived from 6-week-old female mice (Figure 1b). In the absence of Cre recombinase, mammary glands derived from ILK conditional mice showed robust ILK staining in the Keratin 8-positive luminal cells (Figure 1b, ILK^{fl/fl} panel). However in presence of MMTV/Cre transgene, the majority of the mammary luminal cells lacked detectable ILK protein. The efficient ablation of ILK in the luminal epithelial cell population was further correlated with efficient expression of Cre recombinase in the mammary epithelium (Figure 1b, ILK^{fl/fl} Cre panel). To ascertain whether loss of ILK function in mammary epithelial compartment was associated with any developmental defect, we next assessed whether the initial stages of ductal outgrowth were affected by performing whole-mount analyses on both control ILK^{fl/fl} or MMTV-CRE/ILK^{fl/fl} mammary glands from 5 to 8 weeks of age (Supplementary Figure S1A). Given the documented mosaic expression of Cre in the mammary gland of the MMTV/Cre strain, we also introduced into these crosses, the Cre-inducible ROSA26 β -galactosidase reporter (Soriano, 1999). Although the whole-mount analyses revealed no obvious defect in the initial stages of ductal outgrowth (Supplementary Figure S1A), comparison of β -galactosidase staining of the MMTV-CRE/ILK^{fl/fl} mammary glands between 6- and 8-week-old samples revealed that extent of excision was

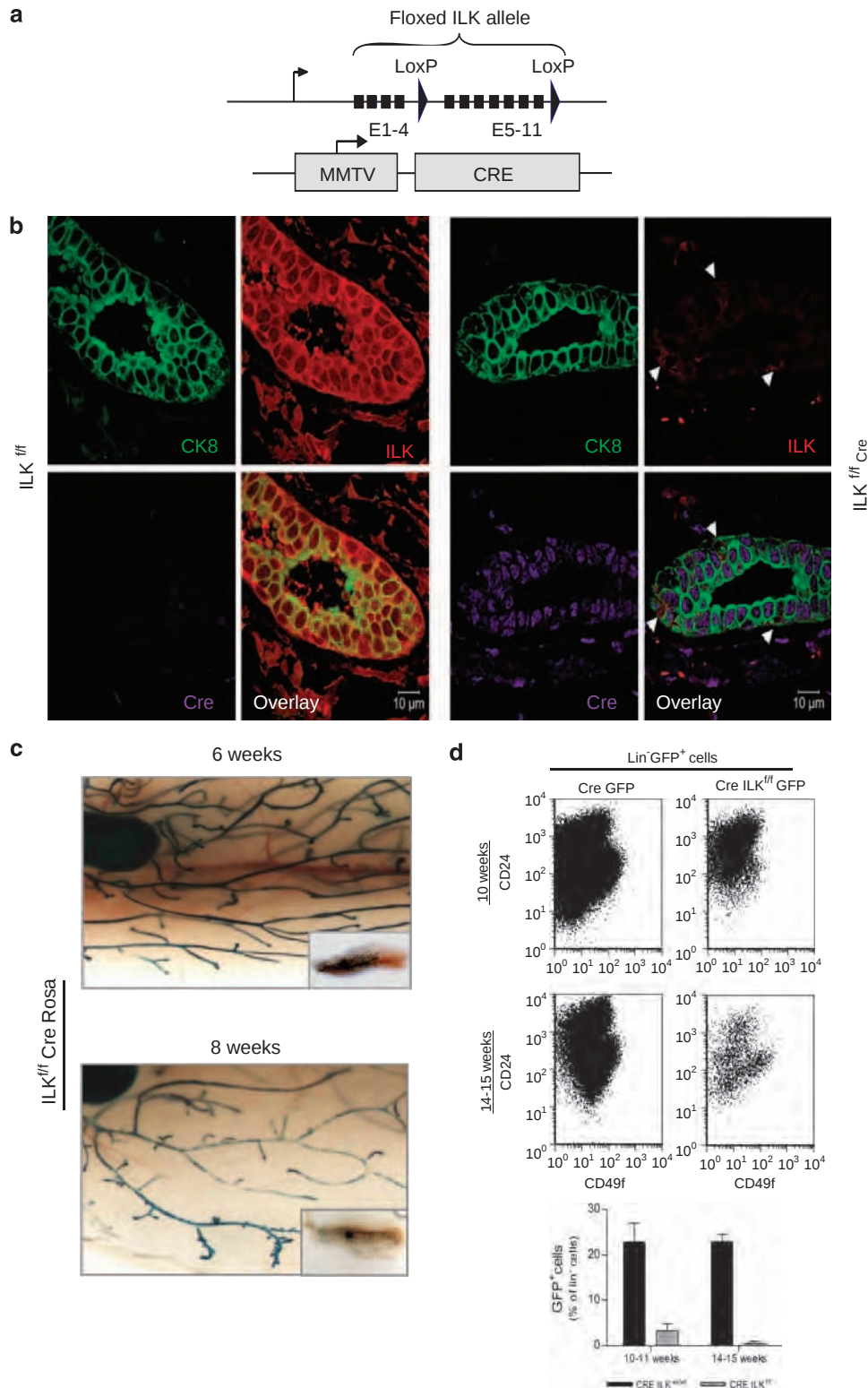
dramatically reduced during these two time points (Figure 2c). These observations indicate that the ILK-defective epithelial cells are selectively lost during these two developmental time points. To monitor precisely the extent of the disappearance of ILK-deficient cells in the mammary gland later in the development, we performed FACS analysis on Cre/ILK^{fl/fl} mammary glands also expressing a Cre-inducible green fluorescent protein (GFP) marker (Kawamoto *et al.*, 2000). Consistent with previous results, at 10 weeks of age, although 20% of the mammary gland epithelial cells (defined as Lin⁻) were GFP-positive in control mammary glands, only 4% of epithelial cells were GFP-positive in a Cre/ILK^{fl/fl} genetic background. This amount was further reduced at 15 weeks (Figure 2d). Collectively, these observations suggest that ILK-deficient mammary epithelial cells are selectively lost during these early stages of mammary gland development.

Previous studies have shown elevated expression of ILK in number of human cancers (McDonald *et al.*, 2008a). Consistent with this view, immunohistochemical analysis of mammary tumors derived from transgenic mice expressing activated ErbB2 under the transcriptional control of the MMTV promoter with ILK-specific antibodies revealed that they expressed elevated levels of ILK protein (Figure 3a, NIC ILK^{wt/wt} panel). To determine if ILK expression is required for the induction of mammary tumors in these MMTV/activated ErbB2 transgenic mice (NIC strain), we introduced the conditional ILK alleles into this strain. We have previously shown that the MMTV/NIC strain develops metastatic mammary tumors with comparable latency to other MMTV/activated ErbB2 strains (Ursini-Siegel *et al.*, 2008). Because NIC transgene also co-express Cre recombinase due to the presence of internal ribosome entry site (IRES), mammary epithelial cells expressing activated ErbB2 will excise the conditional ILK allele (Ursini-Siegel *et al.*, 2008). Thus the coupled expression of ErbB2 and Cre recombinase precludes the possibility of obtaining ErbB2-expressing Cre-negative 'escapee' populations that could possibly contribute to the tumor. Indeed, we successfully showed that this approach results in complete ablation of targeted allele in NIC mice harboring either Stat3 or ShcA conditional alleles (Ursini-Siegel *et al.*, 2008; Ranger *et al.*, 2009).

Using this approach, we generated cohorts of female mice expressing the NIC transgene that were either homozygous or heterozygous for the conditional ILK allele. Heterozygous mice developed multifocal mammary tumors with a similar latency period as that of wild-type controls; homozygous mice, however, developed focal mammary tumors only after a long delay and with dramatically decreased penetrance (Figure 2a). Significantly, only 56% of female cohort carrying both conditional alleles developed mammary tumors after a year observation period. The observed delay in ErbB2-induced tumors in mice homozygous for the conditional ILK alleles was further correlated with the efficient Cre-mediated excision of the conditional ILK alleles (Figure 2b, Tumor 1–6), and marked reduction of ILK

protein levels in premalignant mammary epithelium (Figure 2c) or ILK-deficient tumors from the NIC strain (Figure 3a, NIC ILK^{+/+} panel). Given the dramatic reduced incidence of tumor induction and focal nature of these tumors, these observations suggest that ILK has a critical role in ErbB2-induced tumor progression.

To ascertain whether the block in tumor progression was due to decrease in proliferative capacity, we next investigated whether pre-malignant mammary epithelium expressing activated ErbB2 was compromised in its proliferative status. To accomplish this, we subjected sections derived from premalignant mammary glands at



8 weeks of age to immunohistochemistry analyses with proliferative marker Ki-67 and bromodeoxyuridine using Ki-67 (Figure 2d) or bromodeoxyuridine-specific antibodies (Supplementary Figure S2). To identify those cells that had undergone Cre-mediated recombination of the conditional ILK allele, we also co-stained these sections with antibodies directed toward Cre recombinase. The results revealed that Cre expressing mammary epithelium derived from NIC mice bearing both ILK conditional alleles were severely compromised in their proliferative capacity compared to their wild-type counterparts (Figure 2d, Supplementary Figure S2). Indeed, quantitative evaluation of number of independent sections revealed that ILK-deficient mammary epithelial cell exhibited a 10-fold reduction in their proliferative capacity as indicated by their Ki-67 to Cre expressing ratio (Figure 2d, right panel). These results suggest that targeted disruption of ILK leads to profound proliferative deficit indicating that the block in mammary tumor induction may be partly due to the decreased proliferative capacity of these cells.

ILK-deficient tumor cells have regained their proliferative signaling capabilities

Though mammary-specific deletion of both alleles of ILK resulted in an increased latency period and reduced tumor penetrance in the MMTV-activated ErbB2 mice, focal mammary tumors were eventually observed in 56% of susceptible female animals. To confirm that loss of ILK alleles correlated with loss of ILK protein, we performed immunohistochemistry on ILK-deficient tumors with ILK antisera. Although ILK staining was observed in stromal tissues (Figure 3a), the bulk of the Keratin 8-positive tumor epithelia bearing both conditional ILK alleles failed to stain with ILK (Figure 3c) indicating that these ErbB2-induced tumors were ILK deficient.

To ascertain whether the ILK-deficient tumors exhibited proliferative defect, we next assessed the proliferative status of these tumors by immunostaining with both Cre- and Ki-67-specific antisera. By contrast to premalignant ErbB2-expressing tissue that exhibited a profound proliferative block (Figure 2d, Supplementary Figure S2), the ErbB2-expressing tumors exhibited a high proliferative capacity despite the absence of ILK protein (Figure 3b). These observations suggest that

these ErbB2 ILK-deficient tumor cells have selected alternative signaling pathways that circumvented the requirement of ILK for epithelial cell proliferation.

To identify the nature of alternative signaling network, we next examined whether the ILK-deficient tumors exhibited any obvious defects in signaling pathways that are thought to be regulated by ILK. To accomplish this, we used both reverse-phase protein analyses and immunoblot analyses with phospho-specific antibodies directed at these key downstream signaling molecules (Hennessy *et al.*, 2007). The results revealed that the rare ILK-deficient tumors that arise did not show a significant decrease in signaling through any of these core signaling pathways (Supplementary Figure S3 and S4). Taken together, these observations suggest that ILK-deficient tumors have adapted to induce these signaling pathways in an ILK-independent manner.

ILK deletion impairs signaling and metastasis of ErbB2-expressing tumor cells

Another important function of ILK is to modulate integrin-dependent adhesion (Dedhar *et al.*, 1999). To assess whether activation of integrin-dependent signaling was affected by deletion of ILK, we next performed immunoblot analyses with a number of antibodies directed against integrin-coupled signaling molecules, including paxillin and Cas (Figure 4a). The results revealed that the tyrosine phosphorylation levels of Cas and paxillin were dramatically decreased in ILK-deficient ErbB2-expressing tumors (Figure 4a). The differences in tyrosine phosphorylated Cas and paxillin were not due to changes in protein levels. In fact, the levels of p130Cas were considerably higher in the ILK-deficient tumors (Figure 4a), arguing that tyrosine phosphorylation of p130Cas may be important in p130Cas protein stability. These data suggest that ILK behaves as a scaffold coupling of p130Cas and paxillin to activated c-Src (Figure 4a). Interestingly, the ILK-deficient ErbB2-expressing tumors have a dramatic increase in the tyrosine phosphorylation levels of ErbB3 as compared to wild-type tumors (Figure 4a). Given the documented ability of ErbB3 to activate the phosphatidylinositol-3-kinase signaling pathway, it is conceivable that this selective upregulation of ErbB3 signaling functionally compensates for lack of ILK function. Consistent with this, we observed that despite the tumor

Figure 1 ILK-deleted epithelial cells are selectively lost during mammary gland development. Mice harboring MMTV-CRE genotype in a homozygous background for the ILK flox allele were generated to assess the impact of ILK deletion on mammary gland development. (a) ILK^{lox} allele and MMTV-CRE transgene are represented. (b) Immunohistochemistry (IHC) staining for ILK (red), Cre (violet) and cytokeratins 8 (CK8; green) was performed on mammary glands collected from ILK^{+/+} and ILK^{+/+} CRE mice at 6 weeks of age. White arrowheads indicate the presence in the ILK^{+/+} CRE mammary gland of CRE-negative epithelial cells that still stain positive for ILK. (c) MMTV-CRE/ILK^{+/+}/ROSA26 β -galactosidase mammary glands from 6- to 8-week-old mice were stained for β -galactosidase activity to evaluate the amount of ILK-deleted cells at these two stages of development. (d) Mice harboring the CRE transgene, as well as a GFP recombination marker transgene, in a wild-type or a homozygous background for the ILK flox allele were generated and all 10 mammary glands were collected. Mammary gland cells were single cell dissociated and stained with antibodies against either hematopoietic lineage (Lin)-specific markers (CD31/CD45/Ter119), the epithelial marker CD24 or the α 6-integrin (also named CD49f). The percentage of GFP-positive cells in the Lin⁻ cell population was assessed and represented in function of CD24 and CD49f expression. Results shown in the histogram (lower panel) are representative of two independent experiments performed with two mice for each age and each genotype.

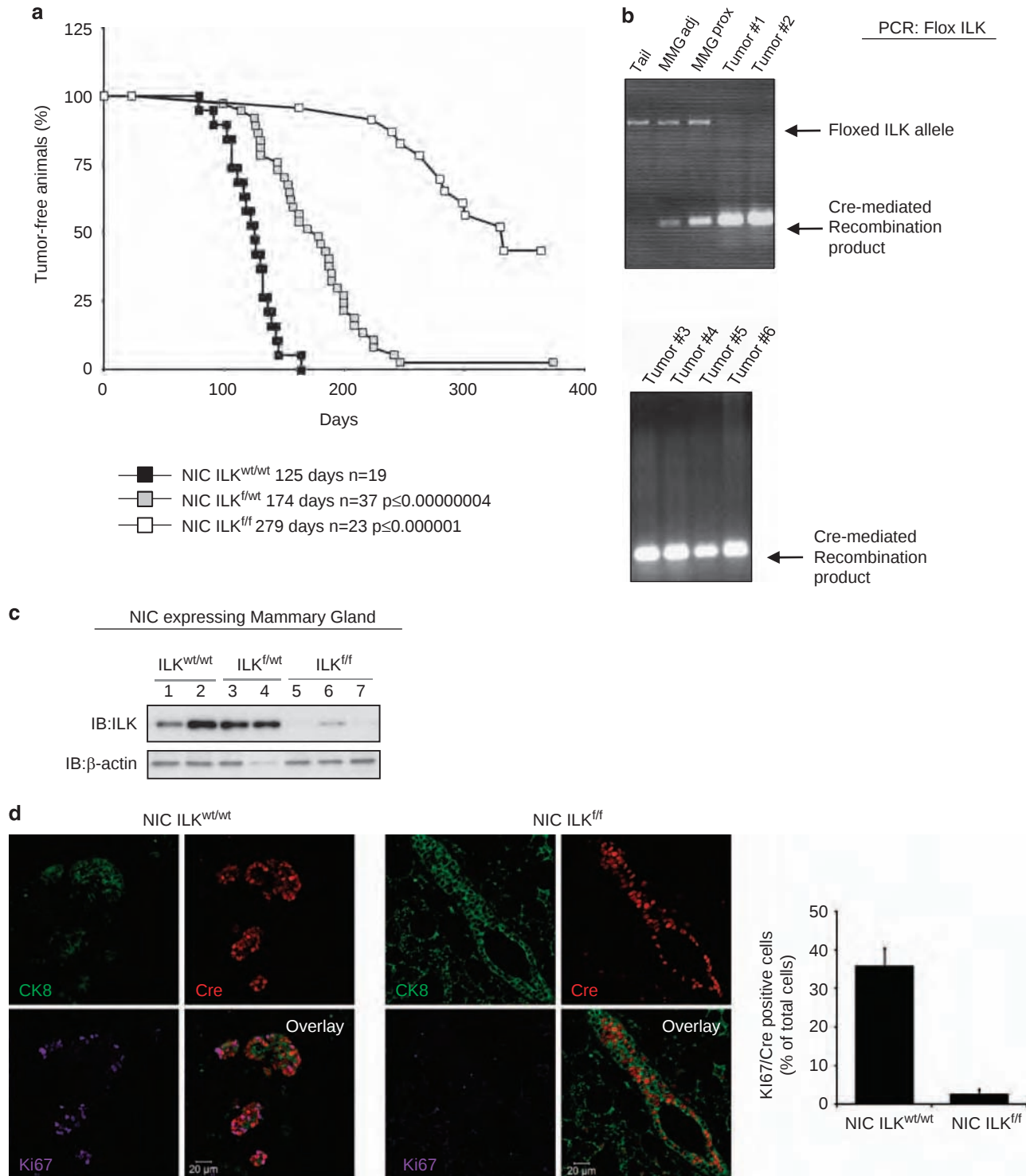


Figure 2 ILK deletion delays Neu/ErbB2-initiated mammary gland tumorigenesis. **(a)** Kaplan–Meier analysis of tumor onsets. Control NIC ILK^{wt/wt} mice vs NIC ILK^{f/wt} vs NIC ILK^{f/f} ($P < 0.000001$). Median tumor onsets values are indicated for the mice that developed tumors. Only 10 out of 23 NIC ILK^{f/f} mice developed tumors. Overall, 13 NIC ILK^{f/f} tumors were collected. **(b)** The efficiency of the excision of the ILK floxed allele in NIC ILK^{f/f}-derived mammary glands and tumors was assessed by PCR. **(c)** ILK expression was assessed in adjacent mammary glands from NIC ILK^{wt/wt}, NIC^{f/wt} or NIC ILK^{f/f} mice by immunoblot. **(d)** Left panel, immunohistochemistry staining was performed on sections of paraffin-embedded mammary glands from 8-week-old mice with the indicated antibodies and observed by confocal microscopy. The bar graph on the right panel represents the percentage of Cre-positive cells expressing also the Ki-67 proliferation marker. Error bars represent the mean $\pm$ s.e.m. of the percentage of double positive (Ki-67/Cre) cells counted in at least three mammary glands.

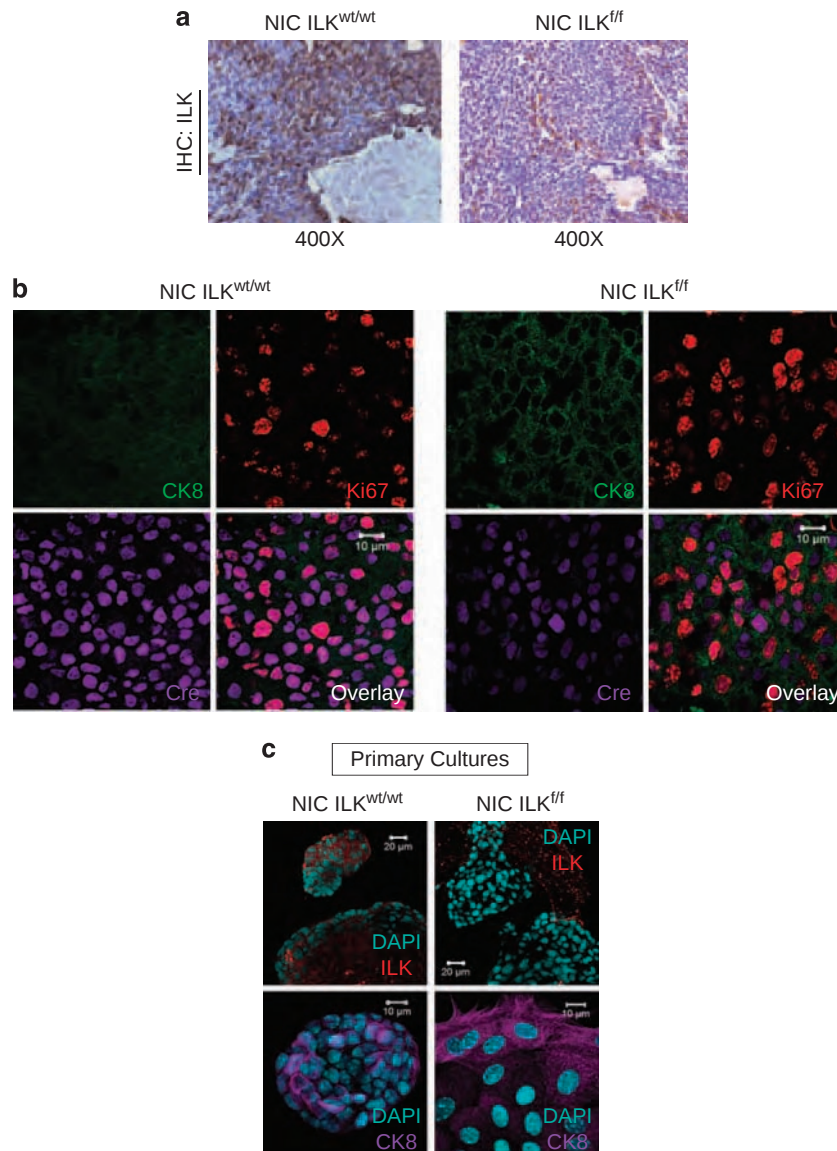


Figure 3 ILK loss does not perturb Neu-expressing tumor cell proliferation. (a) ILK expression level in NIC ILK^{f/f} tumors was assessed by immunohistochemistry on sections of paraffin-embedded mammary tumors with an anti-ILK antibody. (b) Immunohistochemistry was performed with anti-Cre (violet), anti-Ki-67 (red) and anti-CK8 (green) antibodies on sections from paraffin-embedded tumors collected from mice with the indicated genotypes. (c) Representative pictures showing the primary cultures obtained with NIC ILK^{wt/wt} and NIC ILK^{f/f} tumors. Epithelial cells (violet; CK8) are stained for ILK (red).

onset delay and decreased penetrance of the ILK-deficient mice, the tumors had comparable proliferative and growth defects (Figures 3b and 4b).

Given the marked defects in adhesion signaling observed in ILK-deficient ErbB2 tumors, we next investigated whether loss of ILK impacted on the metastatic capacity of the ILK-deficient ErbB2 tumors. Although 67% (8 mice out of 12) of NIC ILK^{wt/wt} mice developed metastatic lung lesions, only 45% (5 mice out of 11) of NIC ILK^{f/wt} and 43% (9 mice out of 21) of NIC ILK^{f/f} mice exhibit metastasis to the lung (Figure 4c). Although the effects of ILK ablation had a modest impact on spontaneous metastasis, injection of ILK-deficient ErbB2 tumors into tail veins of immunodeficient mice revealed a dramatic defect in capacity to

colonize the lung (Supplementary Figure S1C), suggesting loss of adhesion signaling may influence the capacity of ILK-deficient cells to colonize the lung.

Pharmacological inhibition of ILK in established ErbB2 tumors impairs their in vitro invasive properties

Although the above studies suggest that ILK function is required for the initiation of ErbB2 tumor progression, whether ILK function impacted on the invasive properties of established tumor cells was unclear. To further investigate this issue, we developed a novel three-dimensional assay of local tumor dissemination. In this assay, MMTV-ErbB2-derived tumor cells are cultured on a thick bed of Matrigel-reconstituted basement membrane where they form solid, multicellular aggre-

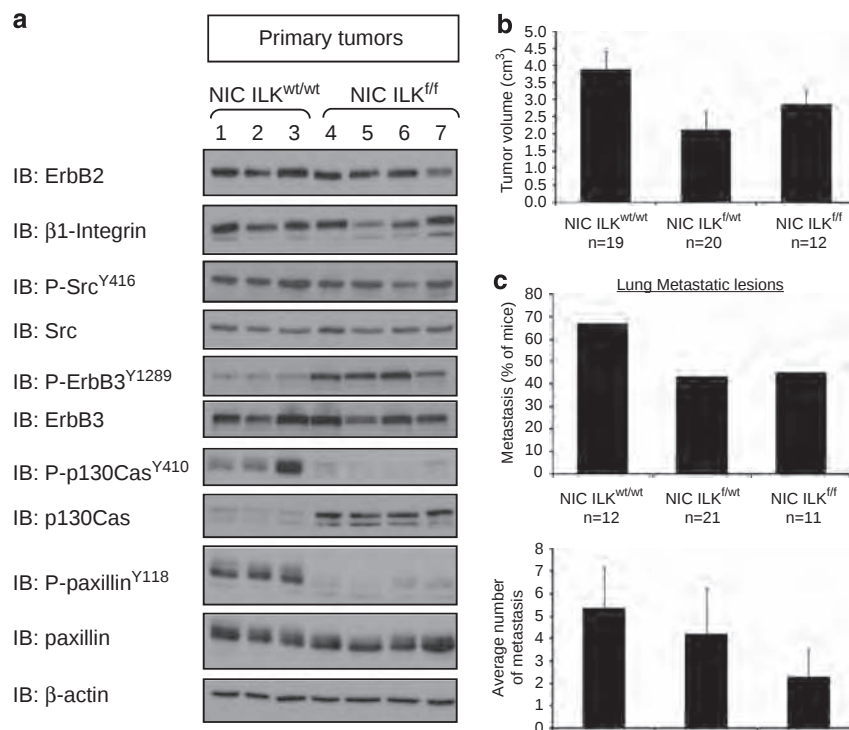


Figure 4 ILK-deleted tumor cells have impaired signaling. (a) Expression of β 1-integrin as well as some of its classical signaling partners was blotted on tumors derived from either NIC ILK^{wt/wt} or NIC ILK^{f/f} mice. (b) Tumor burden of NIC ILK^{wt/wt}, NIC ILK^{f/wt} or NIC ILK^{f/f} mice that have developed tumors. (c) Top panel, percentage of mice from FVB primary genotype (as indicated) that developed lung metastatic lesions. Lower panel, average number of metastatic lesions per lung.

gates that recapitulate the three-dimensional architecture and histological features of MMTV-ErbB2-derived mouse mammary tumors (Figures 5a and c, inset). These structures are noninvasive and resemble ductal carcinoma *in situ*-like lesions, exhibiting well-defined margins and prominent E-cadherin expression at cell–cell junctions (Figure 5c and data not shown). Given the documented pro-invasive role of collagen in advanced human breast cancer (Provenzano *et al.*, 2006), we chose to overlay a collagen I matrix on this Matrigel plug. Once the margins of these ductal carcinoma *in situ*-like acini contact the overlay of connective tissue-derived fibrillar collagen I, individual tumor cells invade and disseminate throughout the collagen I gel (Figures 5b and d). The observed disruption of the acini margins and the loss of cell–cell adhesive junctions in the presence of collagen I resemble the EMT that is correlated with tumor invasion.

To investigate the role of ILK in this *in vitro* invasion assay, we used both RNA interference and small molecule inhibitors directed against ILK. One small molecule inhibitor that has been used to target ILK is QLT0267. This second-generation ILK inhibitor inhibits the kinase activity of ILK and it exhibits a high degree of selectivity against a wide panel of protein kinases (Troussard *et al.*, 2006). Using this *in vitro* invasion assay, we showed incubation of the tumor cells with 10 μ M QLT0267 impaired invasion into the collagen I gel (Figure 5e). To ensure that observed effects on tumor invasion were specific to ILK, we also used RNA interference approach to directly downregulate ILK in

these ErbB2 tumor cells. Consistent with small molecule inhibitor studies, we observed impaired invasion after shRNA-directed knockdown of ILK mRNA and protein expression (Figure 5f, Supplementary Figure S5). Taken together, these observations suggest that ILK has a role in the local invasion of MMTV-ErbB2-derived tumor cells during the early stages of metastasis.

Hematoxylin/eosin-stained sections of untreated control cultures confirmed that collagen I promoted an EMT-like transition to an invasive, elongated tumor cell phenotype (Figures 5g and h). The same transition was not observed, however, when the cells were presented with collagen I in the presence of QLT0267 (Figure 5i). In contrast, disseminated cells in the treated culture appeared small and pyknotic, characteristic of cells undergoing apoptotic cell death (Figure 5i and inset, arrows). The presence of pyknotic nuclei in cells undergoing invasion was also observed after expression of an shRNA construct targeting ILK expression (Figure 5j, arrow). The apoptotic nature of these cells was confirmed by subjecting sections to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. As shown in Figure 5k, disseminated cells in the QLT0267-treated culture stained positive for apoptotic nuclei (Figure 5k, arrows). In contrast, the parental control cells undergoing invasion exhibited little evidence of positive TUNEL staining, consistent with their robust nuclei and abundant cytoplasm (Figure 5l). Quantitative analysis of several independent experiments revealed that $71 \pm 15.5\%$ of the disseminated cells in the QLT0267-

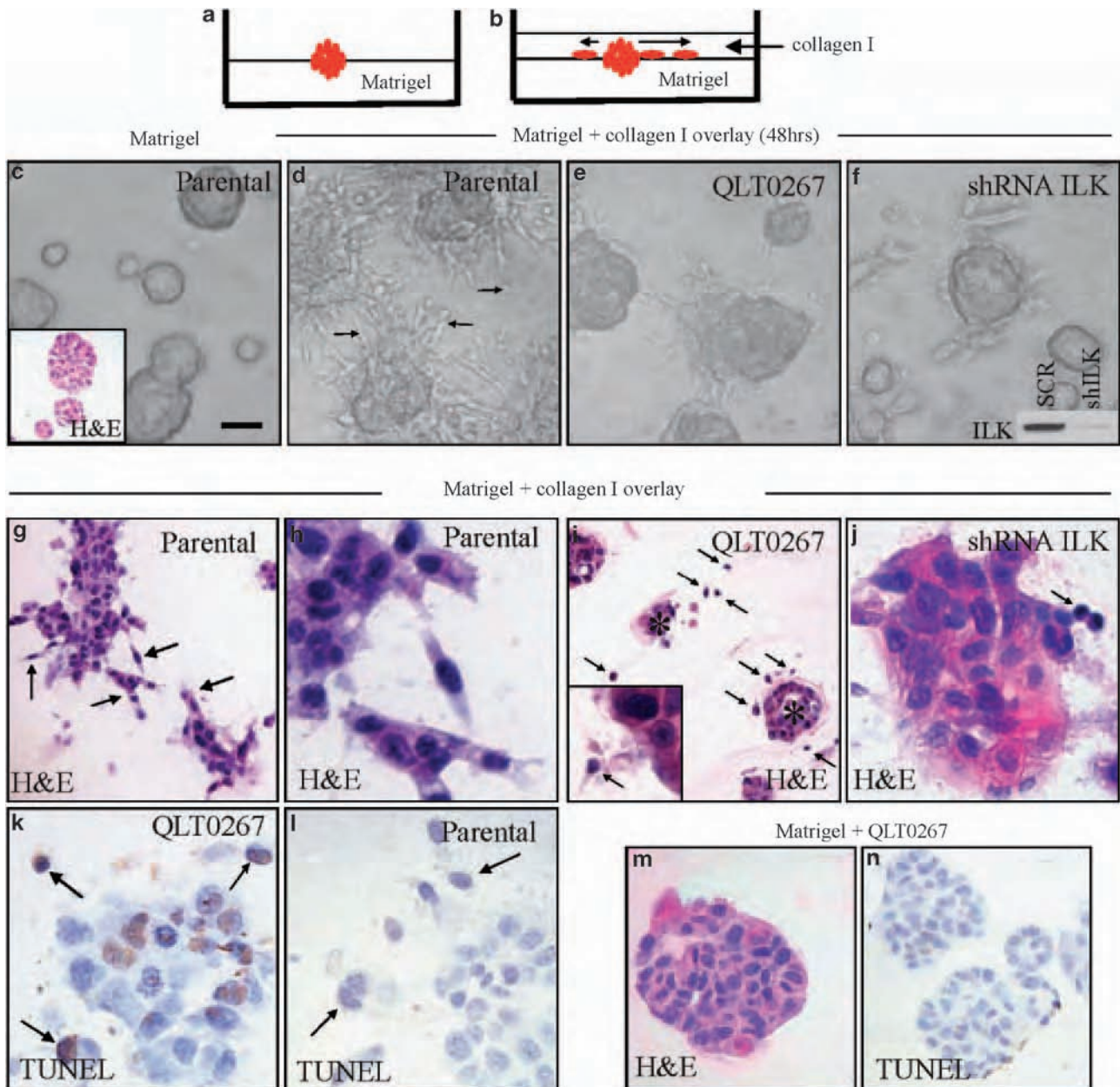


Figure 5 ILK is required for tumor cell invasion *ex vivo*. (a) Experimental setup showing MMTV-Neu-derived tumor cells (red) cultured on a thick (2–3 mm) bed of Matrigel, and (b) after the addition of a collagen I overlay. (c) Phase-contrast image of tumor-like acini formed on Matrigel. Inset shows a hematoxylin/eosin (H&E)-stained section of the Matrigel-embedded acini, revealing the multicellular nature and well-defined margins. Scale bar = 50 μ m. (d) Phase-contrast image of tumor cell invasion (arrows) taken 48 h after collagen I overlay. (e) Phase-contrast image of cells in collagen I after treatment with QLT0267 or (f) Expression of an shRNA construct targeting ILK expression (inset). Panel f is representative of three independent shRNA constructs targeting ILK. (g–j) Hematoxylin/eosin-stained sections of (g, h) parental, (i) 10 μ M QLT0267-treated and (j) shRNA-expressing cells in collagen I after 48 h. Disseminated and invasive cells are marked by arrows. Inset in i shows higher power image of disseminated cell in QLT0267-treated culture. (k, l) TUNEL-stained sections of (k) QLT0267-treated and (l) parental cells in collagen I. Apoptotic cells appear brown. A row of invading cells in l is marked by arrows. (m) Hematoxylin and (n) TUNEL-stained QLT0267-treated cells in Matrigel alone.

treated culture exhibited apoptotic nuclei after 48 h, compared to $12 \pm 6.2\%$ of the disseminated cells in the untreated control culture ($P < 0.0036$; unpaired *t*-test).

Interestingly, QLT0267-treated cells in Matrigel exhibited no evidence of apoptotic cell death by hematoxylin/eosin staining (Figure 5m) or TUNEL staining (Figure 5n) of sections after 48 h incubation. This difference in sensitivity to QLT0267-induced cell

death of cells in Matrigel alone vs the pro-invasive collagen I overlay was not due to differences in the proliferation index (data not shown). Because Matrigel promotes solid tumor-like growth, as opposed to disseminated cells, this observation is consistent with the growth of solid mammary tumors observed in the ILK-deficient ErbB2 tumors. Taken together, these results suggest that ILK-deficient or QLT0267-treated

ErbB2 tumor cells are sensitized to apoptotic cell death in this *in vitro* model of local invasion and metastatic dissemination.

Discussion

An increasing body of evidence implicates integrin signaling as a critical component of both normal mammary gland development and mammary tumor progression. For instance, the targeted disruption of $\beta 1$ -integrin has been shown to affect both the late stages of mammary gland differentiation (Li *et al.*, 2005) and mammary tumorigenesis (White *et al.*, 2004). Central integrin signaling effectors, such as FAK, have also been shown to participate in tumor progression (Lahlou *et al.*, 2007), though these are not involved in normal mammary gland development in either the early (Lahlou *et al.*, 2007) or late (Akhtar *et al.*, 2009) stages of development. Here we show that similar to FAK, ILK has a critical role in the initiation phase of tumor development. Although ILK-deleted cells appear to participate normally in the initial developmental phases of the mammary epithelium, ILK is essential for terminal mammary gland differentiation, because its targeted deletion affects both alveologenesis and lactogenesis (Akhtar *et al.*, 2009). The importance of ILK in these aspects of mammary gland development may reflect its central role in integrating signaling from growth factors and integrin receptors. Indeed, ILK is known to associate with linker molecules such as PINCH that in turn can associate with the receptor tyrosine kinase Nck adaptor protein (Tu *et al.*, 1999). In addition to its critical role in lateral cross talk between growth factor receptors, ILK is also involved in activating key survival and proliferative signaling pathways such as Akt1/PKB α and GSK3 β (Delcommenne *et al.*, 1998; D'Amico *et al.*, 2000; White *et al.*, 2001). In this regard, we have shown that targeted disruption of ILK in the heart results in a dramatic impact on both MAPK and Akt1/PKB α phosphorylation (White *et al.*, 2006).

Although ILK is dispensable for the initial stages of mammary outgrowth, elevated expression of ILK has been implicated in mammary tumor progression. We have previously shown that ectopic expression of ILK results in induction of metastatic mammary tumors that possess the hallmarks of an EMT phenotype (White *et al.*, 2001). Using an ErbB2 transgenic model (Ursini-Siegel *et al.*, 2008), we have shown that mammary epithelial disruption of ILK resulted in a dramatic delay in mammary tumor induction. Analyses of premalignant ErbB2-expressing mammary epithelium lacking ILK function has revealed that this block in ErbB2 tumor induction is associated with profound block in epithelial proliferation (Figure 2d, Supplementary Figure S2). However, the rare ILK-deficient ErbB2-expressing tumors that eventually arose exhibited equivalent proliferative capacity to their ILK proficient counterparts. In addition, phosphorylation levels of both Akt/PKB α and MAPK proteins were comparable in the control and ILK-deficient tumors, suggesting that ILK is not required to activate these two pathways in the ErbB2-driven

tumor signaling network. Consistent with these analyses, reverse-phase protein analyses with a large number of phospho-specific antibodies failed to reveal significant differences in several well-characterized signaling pathways (Supplementary Figure S3). These observations suggest that ILK-deficient ErbB2-expressing tumors evolved alternative mechanisms to activate these critical downstream signaling pathways. In this regard, we have observed an increased activation of the ErbB3 pathway (Figure 4a) that may potentially compensate for loss of ILK function. Indeed, elevated ErbB3 signaling has been implicated in resistance to epidermal growth factor receptor inhibitors in lung cancer (Engelman *et al.*, 2005, 2007; Turke *et al.*, 2010). Given ErbB3's documented role in activation of the phosphatidylinositol-3-kinase signaling pathway (Prigent and Gullick, 1994; Soltoff *et al.*, 1994), elevated activation of phosphatidylinositol-3-kinase pathway is likely responsible for circumventing the requirement for ILK.

Although ILK appears dispensable for the activation of these key proliferative signaling pathways, its loss still induced major defects in other integrin-coupled signaling pathways. In particular, tyrosine phosphorylation of paxillin and p130Cas, but not Src, were compromised in ILK-deficient tumor cells. These observations suggest that ILK may also have a key scaffold role in activation of paxillin and Cas. The loss of adhesion signaling network was further associated with reduction in the spontaneous metastatic burden in these mice (Figures 4b and c). Consistent with this reduction in spontaneous metastasis, injection of ILK-deficient tumor cells into tail veins of immunodeficient mice resulted in a dramatic reduction in the metastatic burden (Supplementary Figure S1B and C). Taken together, these observations suggest that ILK-dependent activation of this key adhesion signaling network may also have a role in the metastatic phase of ErbB2 tumor progression.

This diminished integrin signaling observed in ILK-deficient tumors is reminiscent of PyV mT tumors lacking FAK (Lahlou *et al.*, 2007; Provenzano *et al.*, 2008). These tumors exhibit a complete block in metastasis (Lahlou *et al.*, 2007; Provenzano *et al.*, 2008) that correlates with downregulation of phospho-p130Cas (Provenzano *et al.*, 2008). However, unlike ILK-deficient tumors, disruption of FAK results in the induction of hyperplastic or adenoma lesions that do not progressed to full malignancy (Lahlou *et al.*, 2007; Provenzano *et al.*, 2008).

Previous studies have suggested that metastatic progression may involve transient EMT that facilitates the migratory behavior of cancer cells (Cowin and Welch, 2007). In this regard, it is interesting to note that elevated expression of ILK in either established mammary epithelial cell or primary mammary epithelial cells is associated with an EMT phenotype (Somasisiri *et al.*, 2001; White *et al.*, 2001). In an established mouse model that typically displays a 100% penetrance, we have shown that the deletion of ILK delays the mammary gland tumorigenesis; in fact almost half of the female cohort did not develop any tumor. This phenotype is consistent with the reduced proliferation of these cells

(Figure 2d, Supplementary Figure S2). Consistent with these observations, we have shown that in normal mammary gland development, ILK-deficient epithelial cells are at competitive disadvantage compared to their wild-type counterparts and thus are lost during mammary gland outgrowth (Figures 1c and d). Collectively, these data argue that ILK function has a critical role in the induction of ErbB2 tumors.

Although the studies outlined above clearly show an important role for ILK in the initiation of mammary tumor progression, disruption of ILK function through either a small molecule inhibitor or RNA interference in established ErbB2 tumor cells can impact on their ability to invade in an *in vitro* metastasis assay (Figure 5). However, unlike the proliferative block observed in the initiation phase of ErbB2 tumor induction, the inability to invade correlates with induction of apoptosis (Figures 5j and k). Given the importance of ILK in EMT, it is conceivable that mammary-specific ablation of ILK interferes with this critical phase of tumor metastasis. The association of EMT with metastatic phenotype in ErbB2 tumor induction is further supported by the observation that other modulators of the EMT phenotype such as TGF β can have a profound impact on ErbB2 tumor metastasis (Siegel *et al.*, 2003). Given the importance of metastasis in the morbidity of cancer, the development of therapeutic agents that impact on these key integrin-coupled signaling pathways such as ILK may prove to be attractive option for the treatment of metastatic breast cancer.

Materials and methods

Antibodies

The primary antibodies used in the different experiments performed in this study have been purchased from the following companies: anti-ErbB2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-ErbB3 (Santa Cruz Biotechnology); anti-ILK (Sigma, St Louis, MO, USA); anti-Cre recombinase (Covance, Emeryville, CA, USA); anti-Ki-67 (Novocastra, New Castle upon Tyne, UK); anti-AKT, anti-phospho-AKT^{S473}, anti-p42/44, anti-phospho-p42/44^{T202/Y204}, anti-GSK3 β and anti-phospho-GSK3^{S21/9}, anti-BAD, anti-phospho-BAD^{S136}, anti-Src, anti-phospho-Src^{Y416}, anti-phospho-paxillin^{Y118}, anti-phospho-p130Cas^{Y410} and anti-phospho-ErbB3^{Y1289} (all from Cell Signaling, Danvers, MA, USA); anti- β -actin (Sigma) and anti-cyclin D1 (Santa Cruz Biotechnology). The antisera directed against β 1-integrin, paxillin, p130Cas and anti-bromodeoxyuridine have been purchased from BD Biosciences (San Jose, CA, USA). Guinea pig polyclonal anti-cytokeratin 8/18 was purchased from Fitzgerald (Acton, MA, USA). The Alexa Fluor goat anti-guinea pig 488; anti-mouse 555 and 633; anti-rabbit 555 and 633 antibodies were from Molecular Probes (Eugene, OR, USA).

Transgenic mice

MMTV-Neu-IRES-CRE (NIC), MMTV-CRE and ILK^{flox} mice were generated and characterized previously (Andrechek *et al.*, 2000; Terpstra *et al.*, 2003; Troussard *et al.*, 2003; Ursini-Siegel *et al.*, 2008). GTRosa26 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The CAG-CAT-EGFP reporter mice were described previously

(Kawamoto *et al.*, 2000; Ahmed *et al.*, 2002). To preclude the possible involvement of genetic background variability, we derived all transgenic mice used from the inbred FVB/N strain. Mammary tumor formation was monitored in virgin mice by weekly palpations. For *in vivo* tumorigenesis assays performed with primary cultured cells, we injected 2.5×10^5 cells in the inguinal fat pad of Ncr nude mice (NIH). Tumor progression was monitored twice a week and animals were killed at equivalent tumor volumes. All animal studies were approved by the Animal Resources Centre at McGill University and complied with the guidelines set by the Canadian Council of Animal Care.

Histological, immunohistochemical and immunohistochemistry analyses

After necropsy, mammary glands, tumors and lungs were fixed in 10% neutral buffered formalin (Surgipath, Richmond, IL, USA) and transferred to 70% ethanol the next day. Samples were then paraffin-embedded and sectioned at 4 μ m. Mammary gland tumors and lungs were identified by microscopic analyses of hematoxylin/eosin-stained sections. Whole-mount and *in situ* β -galactosidase assays were performed on inguinal mammary glands as described by White *et al.* (2004). For metastasis analysis, we observed five step sections separated by 50 μ m and counted extra- or intravascular metastases. For immunohistochemistry or immunohistochemistry analysis, we blocked endogenous peroxidase activity with 3% peroxide hydrogen in methanol. Antigen retrieval was accomplished in citrate buffer by using a pressure cooker (Cuisinart, Wood-Bridge, Ontario, Canada). Sections were then blocked with Power Block Universal Blocking Agent (BioGenex, San Ramon, CA, USA) and incubated in primary antibody as described by White *et al.* (2004). For immunofluorescence analyses, we incubated sections with secondary antibodies for 1 h at room temperature and visualized with a Zeiss (Toronto, Ontario, Canada) LSM 510 META confocal microscope.

Mammary gland cell dissociation, primary cell cultures and FACS analysis

Mammary gland fat pads were chopped three times with a McIlwain Tissue Chopper (Mickle Laboratory Engineering Company, Gomshall, Surrey, UK) set to cut at 100 μ m intervals and the finely minced tissue was transferred to a digestion mix consisting of serum-free Dulbecco's modified Eagle's medium media containing 4 mg/ml collagenase A, 4 mg/ml collagenase B, 4 mg/ml dispase and 0.25 mg/ml hyaluronidase (all enzymes purchased from Roche, Mississauga, Ontario, Canada) for 90 min at 37 $^{\circ}$ C under rocking. Cells were then pelleted by centrifugation and incubated for 4 min in 0.25% trypsin/EDTA (Wisent, St Bruno, Quebec, Canada) at 37 $^{\circ}$ C and then with DNase for an additional 2 min at room temperature in phosphate-buffered saline containing 2% fetal bovine serum. After centrifugation, cells were resuspended in Dulbecco's modified Eagle's medium containing NH₄Cl, incubated for 3 min at room temperature and subsequently diluted with phosphate-buffered saline containing 2% fetal bovine serum. Clumped cells were removed by filtering cells on a 70 μ m cell strained. Cells were then spun and resuspended in phosphate-buffered saline containing 2% fetal bovine serum to get a final cellular concentration of 5×10^6 cells per ml.

PCR

PCR analysis of Cre-mediated excision was performed on total genomic DNA prepared from tail, mammary gland or tumor tissues collected from the different generated mice using a standard protocol. PCR primer sequences and amplification

parameters were described elsewhere (Terpstra *et al.*, 2003; Troussard *et al.*, 2003).

Immunoblot analyses

Flash-frozen tumor pieces were prepared in phospholipase-C- γ lysis buffer (Dankort *et al.*, 2001). Protein amounts were quantified by DC Protein (Bio-Rad, Mississauga, Ontario, Canada). Tumor cell lysates were analyzed by immunoblot. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories) and visualized by enhanced chemiluminescence (Amersham, Baie d'urfe, Quebec, Canada).

Reverse-phase protein lysate microarray

Reverse-phase protein microarray was performed as described previously and used to evaluate the expression and the phosphorylation of 40 distinct proteins (Tibes *et al.*, 2006; Hennessy *et al.*, 2007).

Three-dimensional invasion assay

Primary cells were prepared from MMTV-Neu-derived mouse mammary tumors by disaggregation in collagenase I and differential centrifugation, as described previously for various mouse mammary tumor cell types (White *et al.*, 2004). The cells were grown for 2 days on 2–3 mm Matrigel (11.8 mg/ml) (BD Biosciences) to establish multicellular acini. After 2 days a 1–2 mm layer of bovine skin-derived collagen I (at 2.3 mg/ml) (PureCol; Inamed, Nutacon BV, Leimuiden, The Netherlands) was overlaid on top of the Matrigel-embedded acini. For ILK inhibition, we pre-incubated culture with 10 μ M QLT0267 for 30 min before addition of collagen I. QLT0267 was subsequently included in the growth media and collagen I gel. For hematoxylin/eosin staining, we fixed cultures overnight in buffered formalin, then paraffin-embedded and sectioned them at 3 μ m. TUNEL assay for apoptosis was performed according to manufacturer's instructions for the Apoptag kit (Chemicon, Temecula, CA, USA).

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shRNA-mediated ILK knockdown

pLK0.1 lentivirus vectors expressing independent shRNA sequences targeting murine ILK were purchased from Open Biosystems (Huntsville, AL, USA). Clone ID for five independent shRNA sequences are the following: TRCN0000022514, TRCN0000022515, TRCN0000022516, TRCN0000022517 and TRCN0000022518. Before use in three-dimensional cultures, cells stably expressing the shRNA sequences were established by selection in puromycin, followed by confirmation of ILK protein knockdown by western blot analysis (Supplementary Figure S5).

Statistical analysis

Kaplan–Meier analysis was used to analyze mouse tumor onsets.

Conflict of interest

The authors declare no conflict of interest.

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