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14. ABSTRACT

Src kinases represent a family of non-receptor tyrosine kinases that mediate a number of signaling pathways and cell processes that become deregulated during the tumorigenic process such as cell proliferation, growth, motility, and survival. Since its discovery about 35 years ago as the "first known oncogene," the scientific literature has focused on the mutant, oncogenic form of c-Src (v-Src) and its effects in the transformation of fibroblast cells. While these studies have gained insight into several aspects of Src signaling and biology, the role of endogenous c-Src in mammary epithelial cells remains unclear. This proposal aims to use various mammary carcinoma cell lines to examine the role of endogenous Src in two aspects of breast cancer biology, namely, the early event of the loss of proper cell polarity and acini architecture and the later event of increased migration and invasion capabilities. In addition, the proposal will examine the role of Src in more physiologically relevant three-dimensional cultures by employing the commercially available Matrigel ™ which contains components of extracellular matrix proteins to supply mammary tumor cells with proper polarity cues. Using Src-specific pharmacological inhibitors, our laboratory has found that T4-2 cells polarized and formed acinus-like structures in 3-D cultures accompanied by a concomitant downregulation of AKT and ERK phosphorylation. Inhibition of Src in the more invasive MDA-MB-231 mammary carcinoma cells prevents the formation of invadopodia in 3-D cultures. Using the dominant-negative mutant of Src in MDA-MB-231 cells, we have confirmed that Src signaling is required for invadopodia formation. Biochemical and cell biological studies will be aimed at delineating the important downstream effectors of Src involved in loss of acini architecture and gain of invasive capabilities of mammary tumor cells.

15. SUBJECT TERMS
Signal Transduction, Breast Cancer, Src, 3-Dimensional Cultures.

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Introduction:

Signaling by the non-receptor tyrosine Src kinases mediates a number of biological processes such as cell proliferation, survival, migration, invasion, and angiogenesis that become deregulated during tumor progression (Frame, 2003). Studies have shown an elevation in Src activity and/or levels in mammary tumor cell lines and tissue compared to control tissue or non-tumorigenic lines (Egan et. al. 1999, Maa et. al. 1995, Reissig et. al. 2001, Biscardi et. al. 2000). Additionally, transgenic mice models overexpressing the *neu* protooncogene have shown that these mice develop mammary tumors that contain Src activity 6-8 fold higher than adjacent normal tissue **(**Muthuswamy et. al. 1994**).** Despite strong correlation evidence suggesting an important role of Src in tumor progression, the function of Src in mammary carcinoma biology remains unresolved. While numerous studies have shown Src to play important roles in a variety of cellular processes, most of these reports have focused on the effect of exogenous introduction of the oncogenic version of Src, v-Src in fibroblasts (Frame and Fincham et. al 2002, Hauck et. al. 2002). The transforming ability of v-Src stems from mutations in the SH3 domain which disrupt intramolecular interactions within the protein as well as a truncating mutation of the c-terminal 30 amino acids containing the regulatory tyrosine (Martin, 2004). These mutations in v-Src render the protein constitutively active and explain the high transformation ability of v-Src. However, there are no reports that these mutations occur in endogenous c-Src in mammary carcinoma cells. Additionally, the majority of carcinomas arise from epithelial cells and thus fibroblasts may not represent an ideal cell type to address the question of the role of Src in tumorigenesis.

In the context of its microenvironment, epithelial cell are highly polarized and structured. Because these cells make up the organ lining of the body and must tightly regulate the transport of molecules across this lining, this structured architecture is necessary to maintain tissue specific function (Bissell and Raids 2001). During the initial stages of tumor formation, epithelial cells loose their intrinsic polarity and begin to hyperproliferate leading to progressive loss of epithelial architecture. As carcinomas progress they gain the ability to migrate and invade through the underlying extracellular matrix. This feature allows cancer cells to enter the lymphatic system and metastasize at distant sites. Within the mammary glad, epithelial polarity is exemplified by the formation of an extensive ductal network specialized in milk secretion (Radisky et. al. 2003). During puberty, ductal elongation occurs through cell proliferation at specialized structures called terminal end buds (becoming end buds) which are lost at maturity once the ductal network in established within the fat pad. At pregnancy, cells at the end buds begin to proliferate once more and differentiate into secretory lobularaveoli. The cellular structure of the mammary alveolus consists primarily of a two-cell bilayer surrounding a central lumen where milk droplets are deposited for secretion. Specialized luminal cells around the lumen synthesize the milk proteins and are the cellular compartment from which the majority of mammary carcinomas arise. Myoepithelial cells surround the luminal epithelial cells and provide structural support as well as the contractile force necessary for milk secretion. It is now appreciated that in addition to cell intrinsic genetic alterations, changes in the cell's microenvironment and disruption of local cellular architecture can promote tumor progression (Bissell et. al. 2002). This is an important concept since many studies examining tumor cell lines use standard monolayer culturing conditions. The lack of proper polarity signals leaves confounding questions as to the physiological relevance of data obtained from monolayer culture studies and has mitigated the need for more appropriate cell culture systems.

The HMT3352 cell culture series of tumor progression developed by Briand and colleagues (Briand et. al. 1987 and Briand et. al. 1996) allows one to recapitulate some of the events in tumor progression in a more physiologically relevant context. In this system, a non-tumorigenic mammary epithelial cell line (S1) was established under defined media conditions from a woman with a non-

malignant breast lesion. Successive passages of S1 cells in culture allowed for the outgrowth of spontaneously tumorigenic sublines of which T4-2 is one of them that were growth factor independent and contained genetic abnormalities commonly found in carcinomas such as *myc* gene amplification and *p53* mutations. Phenotypically, when both cell lines are grown as standard monolayers (referred to as "2-D cultures") not much difference is observed. When cultured in a laminin rich reconstituted basement membrane such as the commercially available Matrigel ™ (referred to as "3-D cultures"), S1 cells will polarize and form organized multicellular spheroids called acini that resemble the mammary terminal end bud. T4-2 cell continue to proliferate and form large disorganized multicellular colonies that resemble nodules that form during early stages of tumor development .Inhibiting certain cell signaling pathways can induce T4-2 cells to revert and form acini-like structures in 3-D cultures (Weaver et. al. 1997). Figure 1 and Figure 2 in Appendix A provide a cartoon schematic of the HMT-3352 system and how 3-D cultures are made. In addition, use of a reconstituted basement membrane can be adapted to study the behavior of various mammary tumor cells in a context that better approximates tumor progression as is happens in the body. We have chosen two cell lines to study the role Src in tumor progression: T4-2 cells to examine the role Src may have in early events in tumor development such as loss of epithelial architecture and polarity and the highly invasive MDA-MB-231 cell lines to examine the role of Src in later events whereby tumor cells gain the ability of migrate and invade into neighboring tissue.

Body

Data summary presented in initial grant proposal:

In our initial grant proposal, we showed that Src-specific pharmacological inhibitors, PP2 and SU6656 induced pleitropic effects on various mammary carcinoma cell lines suggesting that the role Src played in mammary carcinoma progression was complex. Inhibition of Src activity in T4-2 cells induced these cells to form acinus-like structures as judged by compact colony formation on 3-D cultures and basal localization of the α6 integrin subunit. Treatment of the more aggressive mammary carcinoma cell line, MDA-MB-435S also appeared to induce acinus-like structures as these cells also displayed basal surface restriction of α6 when cultured on 3-D cultures. In contrast, inhibiting Src activity in the invasive MDA-MB-231 cell line did not induce acinus formation. These cells form extensive actin-rich invadopodia-like structures on 3-D culture and Src-activity inhibition prevented the formation of these invasive structures. Additionally, migration of MDA-MB-231 cells into a monolayer scratch was reduced by Src inhibition. These results suggested a role of Src in conferring mammary carcinoma cell with increased migratory and invasive potential in later stage tumors.

Progress summary for previous award year (April 2005-May 2006)

To further confirm that T4-2 cells formed polarized acini upon Src inhibition by PP2, we stained the colonies for additional polarity markers for lateral, and apical distribution. In polarized epithelial cells F-actin localizes to the basolateral surface and the golgi matrix enzyme. GM130 distributes itself apical to the nucleus. In PP2 treated cells, F-actin and GM130 showed proper distribution thus supporting the conclusion that Src inhibition restores proper epithelial polarity in T4-2 cells (see Appendix A, Figures 3 and 4).

While the pharmacological studies described above suggest important roles of Src activity in loss of cell polarity and increased migration and invasion, the potential of the inhibitors' non-specific effects makes it difficult to attribute a specific role to Src in these processes. Indeed, for the initial experiments presented in this proposal, we used 10 μM of PP2 based on the studies utilizing PP2 whereby the inhibitor showed maximal cell biological effect between 10 and 50uM (Nam, et. al. 2002). We performed a series of experiments where the concentration of PP2 was titrated down to 1uM to examine acinus formation. While non-specificity is a constant issue in reports utilizing small molecule inhibitors, we begin to observe acinus formation of T4-2 cells in 3-D cultures beginning at 1uM and reaching a near maximum at 2uM, which is below the PP2 concentrations normally used in the majority of previously published studies (see Appendix A, Figure 5). This makes us confident that the effects observed with PP2 are indeed Src-specific. To confirm that Src activity is indeed inhibited at these lower concentrations of PP2, *in*-vitro kinase assays using purified Src and enolase as a substrate were used. We found that 2uM of PP2 did inhibit Src kinase activity under *in vitro* conditions (see Appendix A, Figure 6, top panel). To test whether Src activity is inhibited *in-vivo* at these lower concentrations, cell lysates from T4-2 3-D cultures +/- 2 or 10uM of PP2 were analyzed for levels of activated Src (phosphoSrc418Y). We found decreased levels of phopshorylated Src at 2uM and 10uM in 3-D cultures confirming the *in vitro* kinase data (see Appendix A, Figure 6, bottom panel). Interestingly, 2uM of PP2 was not sufficient to downregulate Src activity in T4-2 cultured on standard 2-D monolayer cultures supporting an important role for Src in the disruption of epithelial polarity and acinus formation.

We further examined a potential mechanism for the restoration of acini architecture upon Src inhibition by analyzing the activity or levels of potential downstream effectors. Liu and colleagues (Liu et. al. 2004) showed that inhibition of the PI3K pathway with the LY290002 inhibition T4-2 cells was sufficient to induce these cells to form acini in 3-D cultures. Additionally, Debnath and colleagues (Debnath et. al. 2003) showed that overexpression of a constitutively membrane bound form (and thus constitutively active) of a PI3K downstream, AKT, in a non-tumorigenic mammary

epithelial cell line, MCF-10A, led to luminal filling in 3-D induced acini. AKT activation thus represents an important signaling molecule in the progressive deterioration of acini architecture during tumorigenesis. We therefore examined whether Src inhibition led to a downregulation of AKT activation by running western blots on lysates from T4-2 cells cultured on 3-D matrices and treated with either DMSO as a vehicle control or with varying concentrations of PP2; the blots were then probed with anti-phospho AKT (phospho Serine 473). We found a dose dependent decrease in AKT phosphorylation upon PP2 treatment (see Appendix A, Figure 7 and 8 left). Whether the downregulation of PI3K/AKT signaling is required for acini formation induced by Src inhibition is not known. We have stably expressed the myristoylated form of AKT1 in T4-2 cells and are currently assessing whether the myristoylated AKT1 protein can overcome acini formation by Src inhibition. We also plan to use the non myristoylated activated form of AKT as a control to show that that effect is due to activated AKT1 specifically and not due to an artifact caused by secondary effects of overexpressing an artificially membrane bound mutant of a protein. Additionally, we would also like to examine whether activated forms of AKT2 can disrupt acinus formation as several reports have shown isoform-specific functional role between AKT family members (Yoeli-Lerner et. al. 2005, Irie H.Y. et. al. 2005**).** Wang and colleagues (Wang et. al. 1998) found that inhibition of the epidermal growth factor receptor (EGFR) or inhibition of the β1 integrin subunit induced acinus formation in T4-2 cells plated on 3-d cultures. Further analysis showed a reciprocal downmodulation of β1 integrin when EGFR activity was inhibited and vice versa suggesting crosstalk between integrin and growth factor signaling in tumorigenic cells. Additionally, inhibition of ERK activity also induced acinus formation in T4-2 cells. To examine whether these events also occur during acinus formation by Src inhibition, we examined the levels of β1, EGFR and phosphorylated ERK in cells treated with PP2. We found that ERK phosphorylation was decreased at 2 and 10uM PP2 (See Appendix A, Figure 8 right. Interestingly, β1 and EGFR levels were not decreased at 2μM but were decreased at 10μM despite acinus formation at 2μM**.** Furthermore, visual examination revealed that acini formed at 2μM were larger (due to more cells per acinus) than acini formed at 10μM suggesting a more pronounced block of cell proliferation at the higher dose of the inhibitor (see Appendix A, Figure 5). We therefore conclude that there are multiple pathways for acinus formation and that downregulation of EGFR and β1 integrin, while sufficient, is not necessary for the formation of these polarized epithelial structures.

To further validate the results obtained from the pharmacological inhibitor studies, we proposed to inhibit Src activity by genetic means using RNAi technology to reduce the levels of Src and introduction of the dominant negative mutant of Src to block Src signaling. We initially proposed to focus on the RNAi approach to downregulate Src levels. However, because of the possible functional redundancy between the three major Src family members, Src, Fyn, and Yes, it may be necessary to downregulate all 3 family members to observe an effect. Despite high amino acid sequence homology, the nucleotide sequence is quite divergent between Src, Fyn, and Yes. This increases the complexity in RNAi oligonucleotide design and delivery and increases the number of potential variables that could negate any observable effect. For these reasons, we decided to focus our efforts on introducing the EGFP tagged-dominant negative (EGFP-dnSrc; K297M, Y527F) mutant form of chicken Src which would inhibit all three family members. Initial transfection efforts using Lipofectamine 2000 ™ on T4-2 cells were unsuccessful in obtaining cells expressing the EGFP-dnSrc construct. Subsequently, we attempted to use retroviral transduction techniques to obtain stable cell populations expressing our EGFP-dnSrc construct and found that T4-2 cells are also difficult to infect with retroviral vectors. Some success was obtained using viral supernatant derived from phoenix amphotropic packaging cell lines stably expressing GFP-dnSrc. However, stable pools of T4-2 cells expressing EGFP-dnSrc did not expand and we were unable to obtain satisfactory T4-2 stable sublines expressing EGFP-dnSrc. This observation lends support to our findings that showed that PP2 and SU6656 reduced cell proliferation. Implicit in these findings is the conclusion that Src

activity is required for cell proliferation in this mammary carcinoma line. We have had some success introducing GFP-dnSrc with the Fugene-6™ (Roche) transfection reagent. While transfection efficiencies remain low in this line, we were able to obtain a small population of cells transiently expressing the construct. Overexpression of EGFP-dnSrc had various phenotypic effects when T4-2 cells were cultured in 3-D matrices. The majority of cells expressing EGFP-dnSrc did not divide in 3-D cultures and remained single-celled entities (see Appendix A, Figure 10). However a small percentage (<1%) did expand and form compact spheroids that resembled acini (see Appendix A, Figures 10 and 11). EGFP alone was transfected into T4-2 cells as control to rule out the possibility that the effect observed is an artifact of EGFP overexpression. We also stained the cultures with an anti-EGFP antibody conjugated to Alexa Fluor 546 to confirm that the fluorescence was EGFP specific and not background fluorescence resulting from fixation/staining conditions (see Appendix a, Figure 11). The cells were not stained for polarity markers so it is difficult to conclude that EGFP-dnSrc restores epithelial polarity. Because it is clear that inhibition of Src inhibits cell proliferation in T4-2 cells, we are now currently developing a T4-2 tet-off inducible system to regulate the levels of EGFP-dnSrc. We propose to culture these cells in 3-D matrices in the presence of doxycycline and allow the cells to undergo several rounds of division before doxycycline withdrawal and monitor the extent of acinus formation and re-establishment of cell polarity. We feel this approach would allow us to make definitive conclusions on the role Src plays in epithelial cell polarity.

We have successfully introduced EGFP-dnSrc in the more aggressive MDA-MB-231 carcinoma cell line. In agreement with the pharmacological studies, stable pools of MDA-MB-231 cells expressing EGFP-dnSrc displayed a pronounced reduction in invadopodia formation in 3-D cultures compared to cells expressing EGFP alone (see Appendix A, Figures 12, 13, and 14). Three clonal sublines derived from stable pools expressing EGFP-dnSrc were established and all three clones showed decreased invadopodia formation in 3-D cultures (see Appendix A, Figures 15). While these cells do not appear to form acini, inhibition of Src did induce formation of large, compact spheroid colonies. Collectively, the data suggests that downregulation of Src activity prevents invadopodia formation and reduces the invasive capacity of MDA-MB-231 cells. We are currently analyzing for potential downstream effectors necessary for invadopodia formation in this system. Our initial studies will focus on the Rho GTPase family members, Rho, Rac, and CDC42 because of the strong body of evidence linking these proteins to the regulation of cell motility and actin dynamics (Wilkinson et. al. 2005 and Sahai, et. al. 2003**)**. We also wish to focus on AKT family members since it is now appreciated that in addition to its role in cell survival, AKT activity is also important in mammary carcinoma cell motility and invasion (Yoeli-Lerner et. al. 2006, Irie H.Y. et. al. 2005). To that end, we plan examine the levels of Rho, Rac, and CDC 42 activation levels using the Rac binding domain of Pak1 fused to GST as an affinity binding reagent (GST-PBD) to monitor levels of Rac and CDC42 GTP loading. Similarly, we will also use the GST-tagged Rho binding domain of rhotekin to monitor GTP-loading of RhoA and RhoC. If the levels of GTP loading is reduced in any of these GTPases, we plan to introduce activated mutant forms of RhoA(Q63L), RhoC(Q63L), Rac (Q61L), CDC42 (G12V) in the clones expressing EGFP-dnSrc and examine whether invadopodia formation is rescued. Results from these studies would give insight into signaling mechanisms involved in Src-mediated invadopodia formation. Invasion and migration assays using transwell chambers will also be used to quantify the migratory and invasive capabilities of MDA-MB-231 expressing EGFP-dnSrc and to validate our observations from 3-D cultures. We will then examine whether constitutive activation of the Rho GTPase family members can restore migration and invasion. To specifically address the question whether Rho GTPase family members play a role in matrix degradation, MDA-MB-231 cell lines expressing EGFP-dnSrc or EGP alone will be plated on coverslips coated with gelatin conjugated to the fluorescent Alexa 488 dye (molecular probes). Matrix degradation can be monitored by the formation of black holes where the cells have degraded the matrix. We hypothesize that MDA-MB-231 cells expressing EGFP-dnSrc will have a reduced capacity to degrade the

extracellular matrix. As with experiments described above, we will subsequently introduce the activated mutants of RhoA, RhoC, Rac, and CDC42 to examine whether the ability to degrade the extracellular matrix is restored. Additionally, parallel experiments will be performed to examine the importance of AKT signaling in Src-mediated invasion and migration processes. The levels of AKT activation will be monitored using anti-phospho AKT in MDA-MB-231 cells expressing either EGFP alone of EGFP-dnSrc in 3-D cultures. If a decrease in AKT phosphorylation is observed, we will introduce the myristoylated and activated mutants of AKT1 and AKT2 in the stable clones expressing EGFP-dnSrc and perform downstream migration, invasion, and zymography assays as described above for the Rho family GTPases. Future experiments will also examine metalloprotease (MMP) expression and activity using western blotting and in-gel zymography assays to assess whether Src regulates MMP function in MDA-MB-231 cells.

Key Research Accomplishments and Conclusions

- Confirmed proper polarity establishment of T4-2 cells treated with PP2.
- Determined expression of EGFP dnSrc can cause cell cycle arrest and induce formation of acinus-like structures in T4-2 3-D cultures but requires final confirmation with tet-off EGFP-dnSrc inducible system.
- Determined PP2 caused downregulation of AKT and ERK activation in T4-2 3-D cultures. Activated mutant forms of AKT and ERK will be used to determine whether these AKT and ERK signaling can disrupt acinus formation induced by Src inhibition.
- Determined that PP2 did not downregulate β1 or EGFR levels suggesting that downregulation of these molecules is sufficient but not necessary of acinus formation
- Expressed EGFP-dnSrc in MDA-MB-231 cells and confirmed inhibition in invadopodia formation in 3-D cultures. Activated forms of RhoA, RhoC, Rac, Cdc42, AKT1 and AKT2 will be used to determine whether activation of these potential downstream effectors can rescue the loss of invadopodia formation induced by Src inhibition.

Reportable Outcomes

- Poster presentation at the California Breast Cancer Research Program (CBCRP) symposium in Sacramento, California-September 9-11, 2005. (see Appendix B for symposium abstract)

References:

Bissell, M.J. and Radisky, D. Putting tumors into context. Nature Rev Cancer. **1**: 46-54. 2001.

Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. The organizing principle:

microenvironmental influences in the normal and malignant breast. Differentiation. **10:** 537-46. 2002 Dec;70(9-10):537-46.

Briand, O., Petersen, O.W., van Deurs, B. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined media. In vitro Cell Del Biol. **23:** 181-188. 1987.

Briand, O. Nielsen, K.V., Madsent, M.W., Peterson, O.W. Trisomy 7p and malignant transformation of human epithelial cells following epidermal growth factor withdrawal. Cancer Res. **57:** 2039-2044. 1996.

Debnath, J., Walker, S.J. Brugge, J.S. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Bio. **163**: 315-326. 2003.

Frame, M. Src in cancer: deregulation and consequence for cell behavior. Biochim Biophys Acta. **160**: 114—130. 2002.

Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, Natesan S, Brugge JS. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol. 2005 Dec 19;171(6):1023-34.

Liu H., Radisky D.C., Wang F., Bissell M.J. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells**.** J Cell Biol. **164:** 603-12. 2004.

Martin G.S. The road to Src. Oncogene. 48: 7910-7. 2004**.**

Nam JS, Ino Y, Sakamoto M, Hirohashi S. Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. Clin Cancer Res. **8:** 2430-2436. 2002.

Radisky D.C., Hirai Y, Bissell M.J. Delivering the message: epimorphin and mammary epithelial morphogenesis**.** Trends Cell Biol. **8:** 426-435. 2003.

Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.Nat Cell Biol. **5:** 711-719. 2003.

Weaver, W.M., Peterson, O.W., Wang, F., Larabell, C.A. Briand, P., Damsky, C., Bissell, M.J. Reversion of the malignant phenotype of human breast in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Bio. **137**: 231-245. 1997.

Wilkinson S, Paterson HF, Marshall CJ. Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. Nat Cell Biol. **7**: 255-261. 2005.

Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A.. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell. 20: 539-550. 2005. Erratum in: Mol Cell. 2006 Apr 7 145.

Appendix A: Supporting Data and Figures

Figure 1: Schematic of HMT-3352 cell culture model described in report

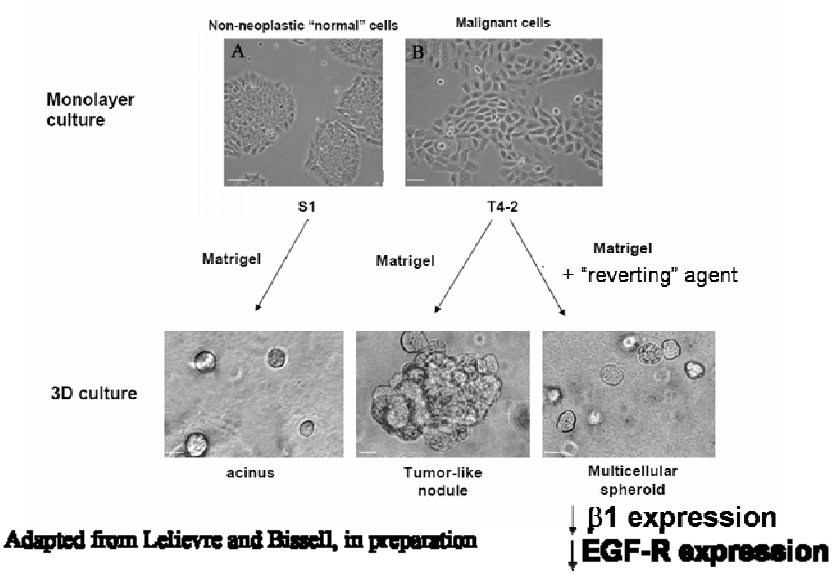


Figure 2: Schematic of cell culture methods described in report.

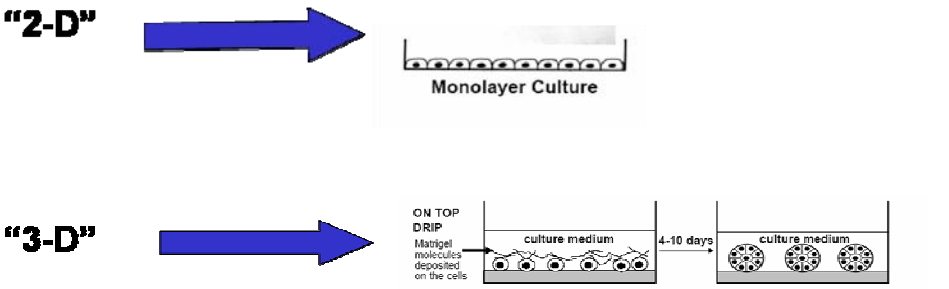
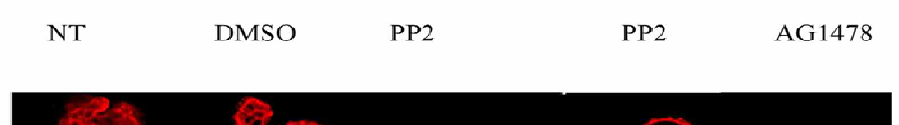


Figure 3: Basal localization of α6 and apical distribution GM130 in PP2 inhibited T4-2 cells in 3-D cultures. T4-2 cells were cultured on chamber slides coated with Matrigel™ for 10days in the presence of 10μM PP2. Cells were then fixed and stained anti α6 followed by anti-rat alexa fluor 546 or anti-GM130 followed by anti rabbit alexa fluor 488. All cells were stained with the nuclear stain DAPI to examine lumen formation. The two columns marked "PP2" represent two different acini displaying polarized structure. AG1478 is an EGFR inhibitor and was used as a positive control for



acinus formation of T4-2 cells in 3-D cultures.

Figure 4: Basolateral distribution of F-actin. 3-D cultures of T4-2 cells treated with PP2 or AG1478 were fixed and stained with either phalloidin 546 or stained with anti-α6 followed with anti-rat alexa fluor 488. AG1478 treatment was used as a positive control for acinus formation in T4-2 cells.

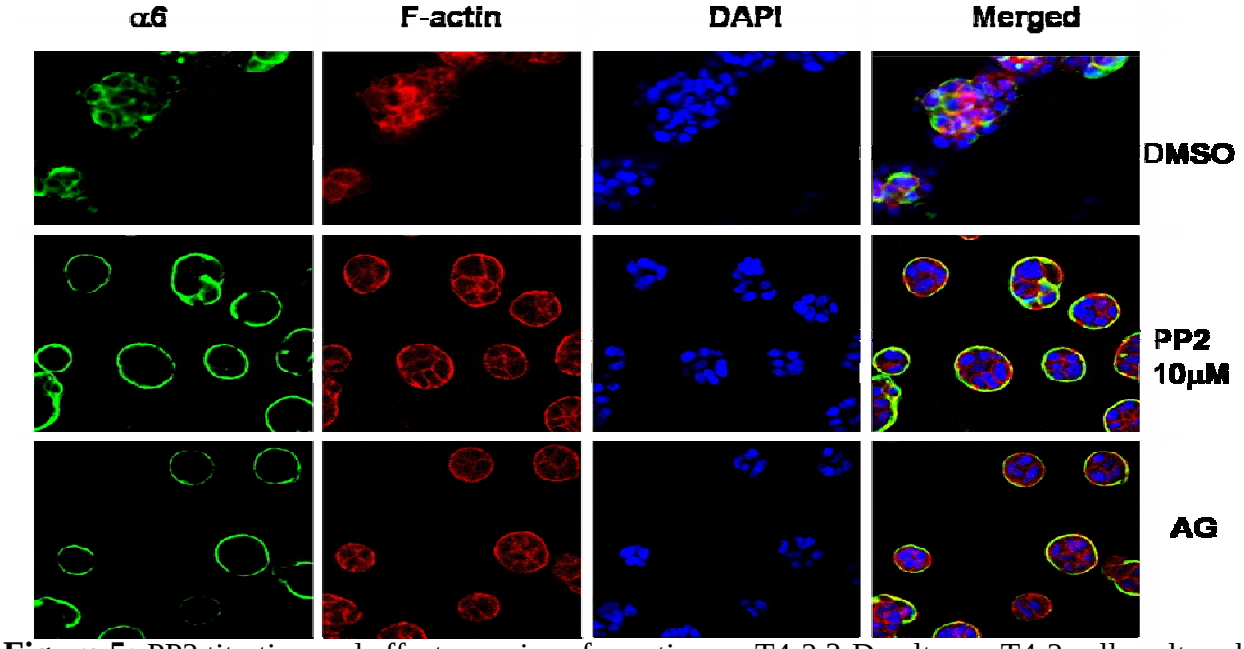


Figure 5: PP2 titration and effect on acinus formation on T4-2 3-D cultures. T4-2 cells cultured in Matrigel ™ were treated with varying amounts of PP2 for 10days followed by phase microscopy analysis. The phase contrast field to the right shows the morphology of the non-tumorigenic S1 cells cultured in 3-D conditions for comparison.

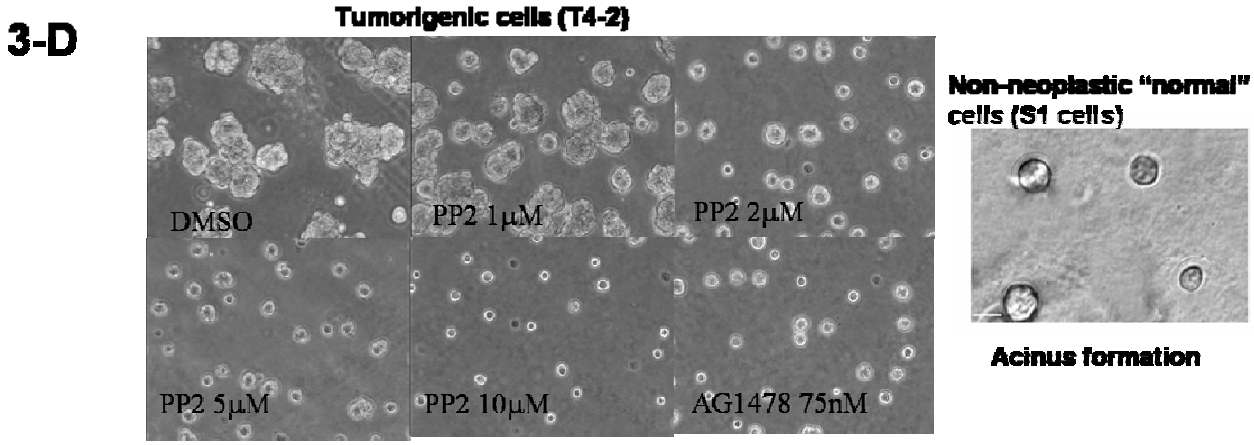


Figure 6: Src inhibition by PP2. Top panel: Recombinant active Src was treated with 2μM PP2 20min prior to the kinase reaction. Acid denatured enolase was used as substrate. The kinase reaction was allowed to for 30 minutes at 30°C before reaction termination and analyzed by western blot with an anti-phosphotyrosine antibody. Control reactions included no ATP to show the reaction was ATP dependent and no recombinant Src. Bottom panel: T4-2 cells were seeded in 2-D cultures for 48hours or 3-D cultures for 10days prior to harvest and western blot analysis with anti-phospho Src (p-Y418). Blots were stripped and probed with anti-Src antibody to ensure equal loading between lanes.

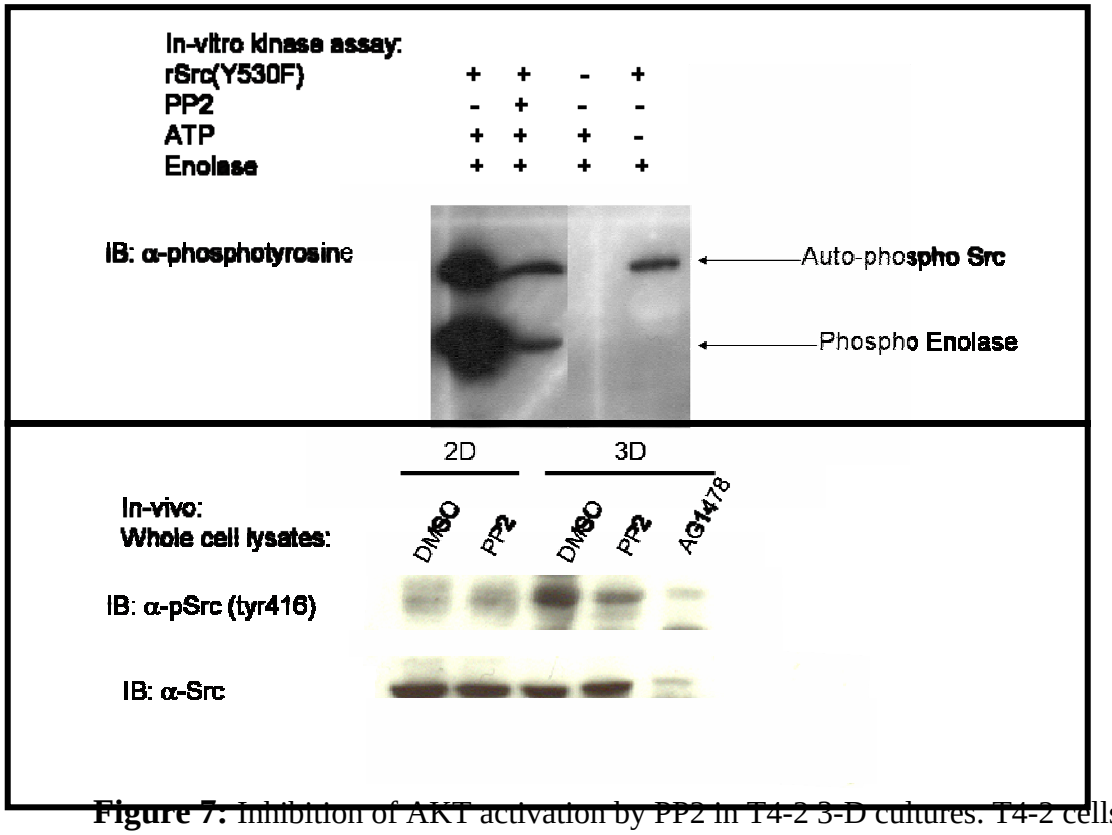


Figure 7: Inhibition of AKT activation by PP2 in T4-2 3-D cultures. T4-2 cells were cultured on 2-D or 3-D cultures for 10 days. Lysates prepared from cultures were analyzed by western blot with anti-phospho AKT (phospho-Ser473). Membranes were stripped and reprobed for total AKT to ensure equal loading between lanes.

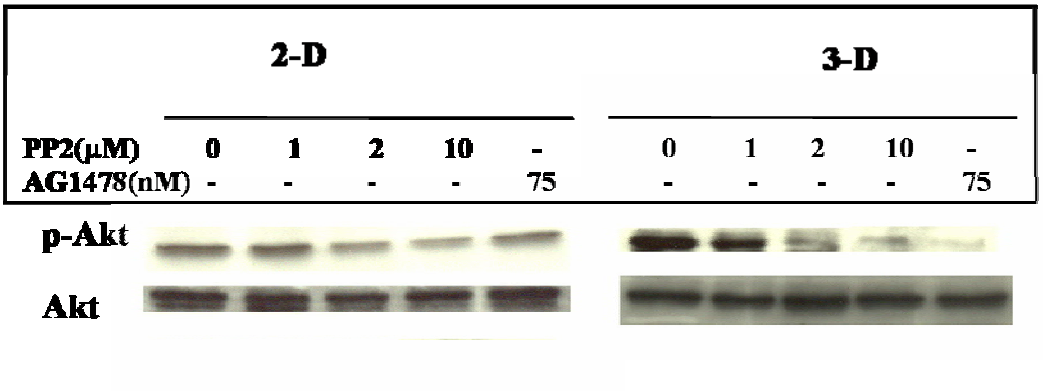


Figure 8: Inhibition of AKT and ERK activation by PP2 in T4-2 3-D cultures. T4-2 cells were cultured on 3-D cultures for 10days. Lysates prepared from cultures were analysis by western blot with anti-phopsho AKT (left) or anti-phospho ERK (right). Membranes were striped and reprobed for total AKT or ERK to ensure equal loading between lanes.

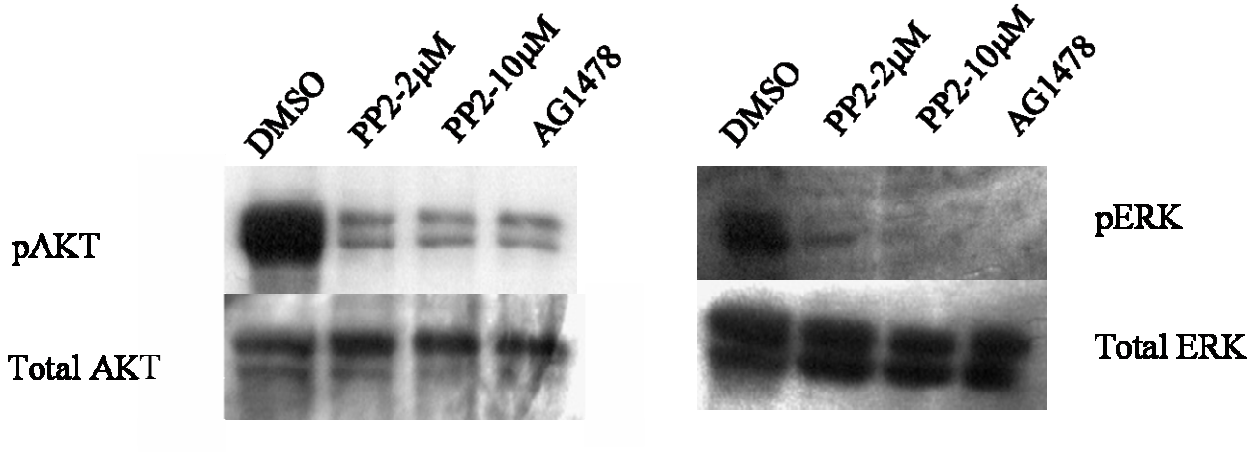


Figure 9: No decrease in EGF-R or β1 integrin in T4-2 cultures treated with PP2. T4-2 cells were seeded in Matrigel™ for 10days (3-D) or as monolayers (2-D) in the presence of 2μM PP2 or 75nM AG1478 (EGF-R inhibitor; positive control for acinus formation). Anti-e-cadherin probing was used as a loading control.

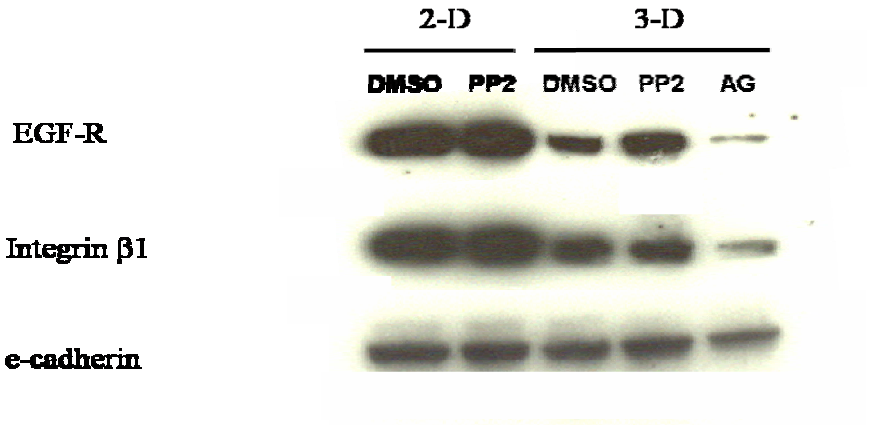


Figure 10: Acinus formation induced by EGFP-dnSrc. T4-2 cells were transfected in monolayer cultures with Fugene-6/EGFP-dnSrc or EGFP DNA complex for 24hours, trypsinized, and seeded in Matrigel™ for 8 days. EGFP fluorescence was examined using standard epifluorescence microscopy

equipped with FITC filters. Representative images of two fields of cells transfected with EGFP alone and 3 fields of cells transfected with EGFP-dnSrc are shown.

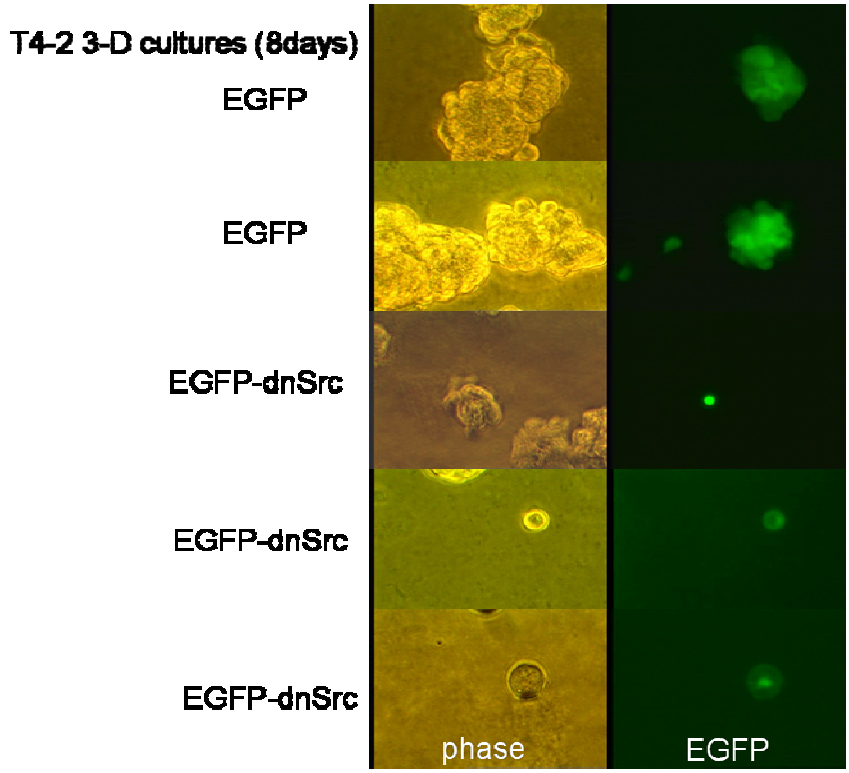


Figure 11: Acinus formation induced by EGFP-dnSrc. T4-2 cells were transfected with Fugene-6/EGFP-dnSrc DNA for 24 hours on monolayers, subsequently trypsinized and seeded in Matrigel™ for 10days.Cultures were fixed and stained with anti-EGFP followed by anti-rabbit Alexa fluor 546. Fluorescence was visualized by epifluorescence microscopy. Representative images of two fields (bottom and top row) are shown.

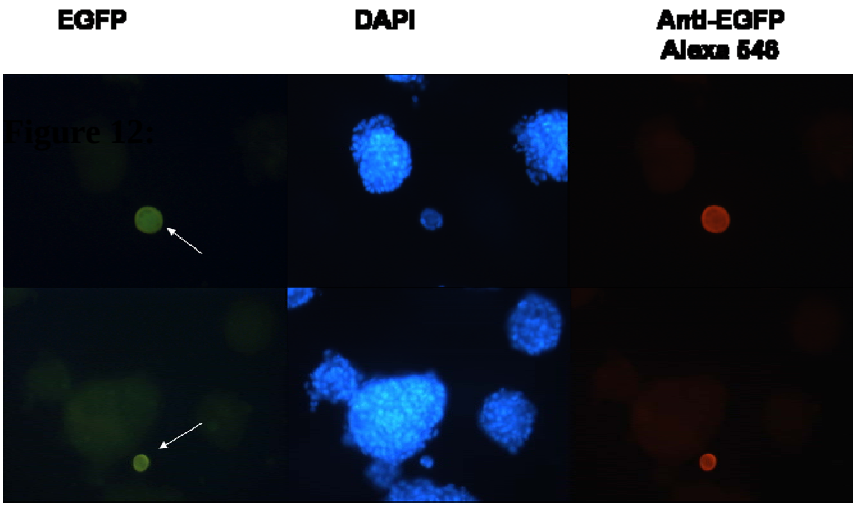


Figure 12:

Figure 12: Inhibition of invadopodia formation by EGFP-dnSrc. Stable pools of MDA-MB-231 cells expressing pBabe-puro empty or pBabe-purp-EGFP-dnSrc were seeded in Matrigel for 8days. Cultures were visualized by phase microscopy at 3, 5, and 8 days. One representative field for each time point is shown.

Figure 13: Inhibition of invadopodia formation by EGFP-dnSrc. Stable pools of MDA-MB-231 cells expressing pBabe-puro empty or pBabe-puro-EGFP-dnSrc were cultured in Matrigel™ for 10days, fixed and stained with phalloidin-546 (Binds to F-actin). Fluorescence was visualized by epifluorescence microscopy. One representative image for each group is shown.

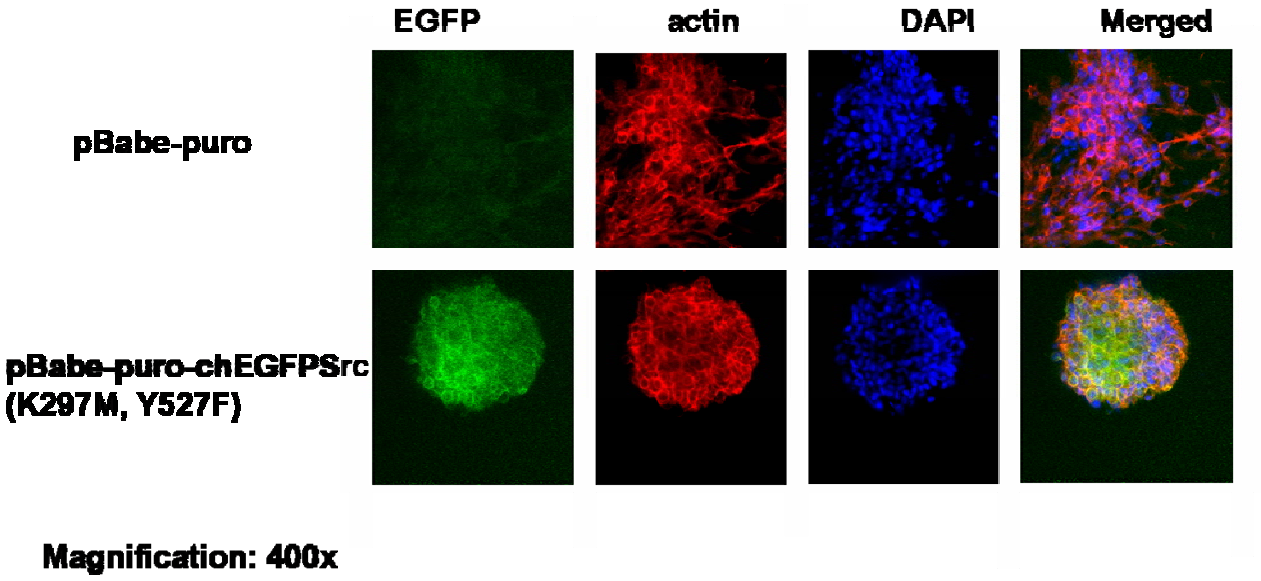


Figure 14: Inhibition of invadopodia formation by EGFP-dnSrc. MDA-MB-231 cells stably expressing EGFP alone, empty pBabe-puro vector, or EGFP-dnSrc were seeded in Matrigel™ for 10days, fixed and stained with phalloidin-Alexa 546.. Cultures were visualized by epifluorescence microscopy. Representative images of one field of EGFP or empty vector and 2 fields of EGFP-dnSrc

(EGFP-dnSrc1, EGFP-dnSrc2) are shown.

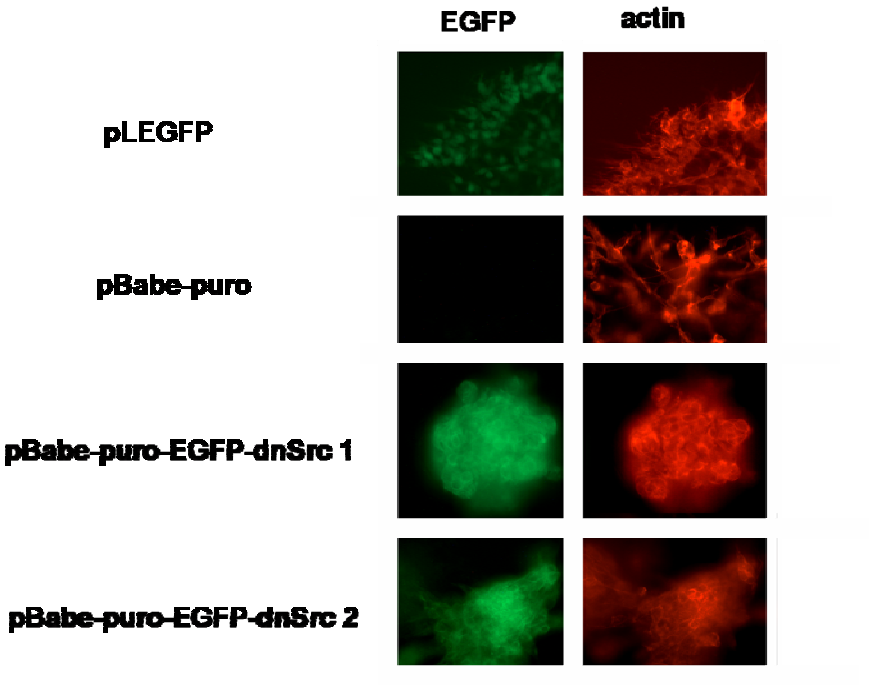


Figure 15: Inhibition of invadopodia formation by EGFP-dnSrc. Clones of MDA-MB-231 cells expressing EGFP or EGFP-dnSrc were cultured in Matrigel™ for 10days. Cultures were visualized by phase microscopy. Two representative images of EGFP-clone 1 and EGFP-dnSrc-clone 1 are shown (data from clones 2 and 3 not shown).

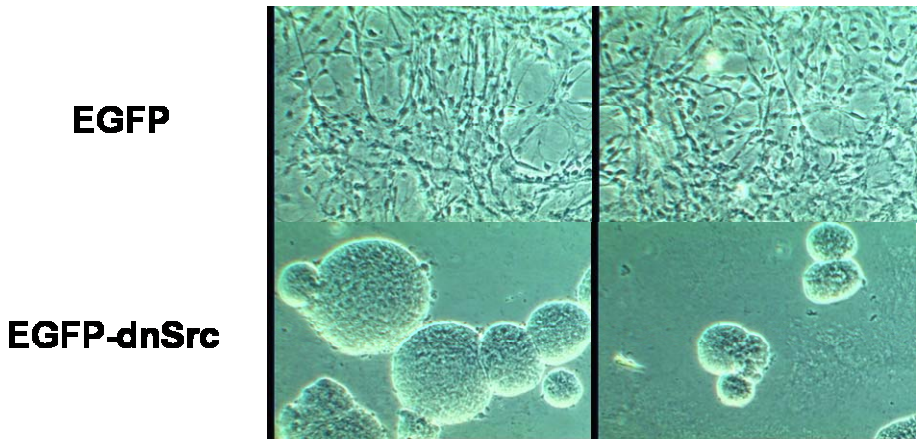
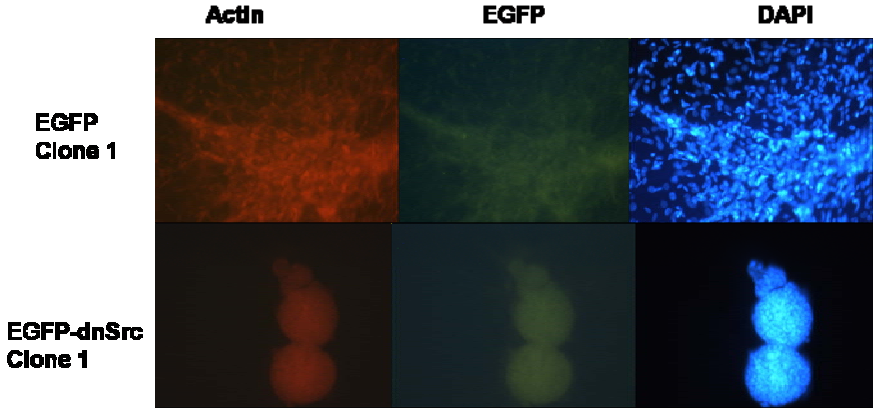


Figure 16: Inhibition of invadopodia formation by EGFP-dnSrc. Clones of MDA-MB-231 cells expressing EGFP or EGFP-dnSrc were cultured in Matrigel™ for 10days fixed and stained for phalloidin alexa fluor 546. Cultures were visualized by epifluorescence microscopy. One representative image of EGFP-clone 1 and EGFP-dnSrc-clone 1 are shown (data from clones 2 and 3

not shown).



Appendix B

Abstract from CBCRP 2005 symposium

Inhibition of the non-receptor tyrosine kinase Src in breast carcinoma cells causes restoration of mammary acinus architecture in three-dimensional basement membrane cultures. Kusdra, L.(1), Crimmins, G.(1), Bissell, M.J. (2), Martin, G.S. (1)
(1) University of California, Berkeley

(2) Lawrence Berkeley National Laboratory

The primary goal of this project is to elucidate the function that the non-receptor tyrosine kinase, c-Src, plays in mammary carcinoma cells. c-Src regulates many signaling pathways that control cell processes such as proliferation, survival, polarity, migration, and invasion. However, although the levels and activity of Src have been shown to be elevated in numerous breast cancer cell lines and tissue samples from breast cancer patients, the specific role that Src plays in mammary cells remains undefined. We have investigated this question by examining mammary cells cultured in a three-dimensional reconstituted basement membrane (Matrigel^{tm}). When cultured in Matrigel, normal mammary cells will polarize and form acinar structures similar to the terminal end buds formed in the mammary tissue during lactation and pregnancy. Tumorigenic cells fail to polarize in Matrigel and will form large, disorganized colonies. Treatment of the tumorigenic cells with compounds that inhibit certain signaling pathways can induce certain cells to revert and form acinar-like structures similar to normal mammary cells.

Using two Src-specific pharmacological inhibitors, we have found that Src inhibition induces reversion of two tumorigenic cell lines (T4-2 and MDA-MB-435S) and formation of acinar structures in 3-D cultures as assessed by the polarized localization of protein markers in these cells. A third, more aggressive and invasive cell line, (MDA-MB-231) failed to form acini in 3-D cultures upon Src inhibition. However, formation of cytoplasmic projections that may be involved in cell motility and invasion was prevented in this cell line when Src was inhibited. Further analysis of reverted T4-2 cells in 3-D cultures has shown that the activation of a kinase involved in tumorigenesis and cell survival, AKT, and expression of a component of a membrane receptor involved in increased proliferation, integrin β1, are downregulated upon Src inhibition. Previous studies have shown that direct inhibition of AKT activation or inhibition of β1 integrin in T4-2 cells can induce reversion in 3-D cultures. Thus, AKT and β1 may represent potential downstream targets of Src in T4-2 cells. We are currently examining other potential downstream signaling targets of Src in order to better understand the mechanism of reversion induced by Src inhibition. In addition we are utilizing DNA recombinant technology to genetically inhibit Src activity in breast carcinoma cell lines to validate the pharmacological studies.

Src plays a role in many biological processes that become deregulated during tumorigenesis and therefore may represent an attractive therapeutic target in the treatment of breast cancer disease. Indeed, Gleevec, a small molecule pharmacological inhibitor of another tyrosine kinase, Abl, that is in clinical use for the treatment of chronic myelogneous leukemia, provides evidence that targeting tyrosine kinases may be a viable therapeutic route in cancer treatment. The results from this study have begun to address the role of Src in breast cancer in a more physiological 3-D culture system. Results from this study may help us better understand the role tyrosine kinases play in breast cancer pathogenesis and may yield clues in the development of more effective therapies.