Review

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Cell-cell and cell-matrix adhesion in survival and metastasis: Stat3 versus Akt

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Abstract: Both cell-cell and cell-matrix adhesion are important for epithelial cell differentiation and function. Classical cadherins mediate cell to cell interactions and are potent activators of the signal transducer and activator of transcription (Stat3), thereby offering survival signaling. While the epithelial (E)-cadherin is required for cells to remain tightly associated within differentiated epithelial tissues, cadherin-11 promotes invasion and metastasis, preferentially to the bone. Cell adhesion to the extracellular matrix is mediated through the integrin receptors that bind to the focal adhesion kinase (FAK)/Src complex, thus activating downstream effectors such as Ras/Erk1/2 and PI3k/Akt, but not Stat3. Therefore, at high densities of cultured cells or in epithelial tissues, co-ordinate activation of the complementary cadherin/Stat3 and integrin/FAK pathways can greatly enhance survival and growth of tumor cells. In neoplastically transformed cells on the other hand, a variety of oncogenes including activated Src or receptor tyrosine kinases, activate both pathways. Still, most single-agent therapies directed against these signaling pathways have proven disappointing in the clinic. Combined targeting of the Src/FAK and Stat3 pathways with inhibitory drugs would be expected to have greater efficacy in inhibiting tumor cell survival, and enhancing sensitivity to conventional cytotoxic drugs for treatment of metastatic disease.

Keywords: Akt; apoptosis; cadherins; Cdc42; EMT; FAK; IL-6; metastasis; Rac; signal transducer and activator of transcription-3.

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Introduction

Both cell-cell and cell-matrix interactions are important in maintaining the functional state of differentiated epithelia in most tissues and organs in the body. Classical cadherins (E and P) are key regulators of adherens junctions which maintain polarity of epithelial cells through cell-cell interactions in the apical region (1). In addition to providing scaffolding structures in cells, cadherin engagement can activate a pathway dependent upon the signal transducer and activator of transcription-3 (Stat3) which is critical for cellular survival (2, 3). In contrast, integrins (β1 and β4 subtypes) are important in anchoring epithelial cells to the underlying extracellular matrix (ECM) substratum, and maintaining cell polarity and function through cytoskeletal signaling (4, 5). Integrin engagement also triggers cell survival, thus preventing apoptotic death of single cells which lack cell-cell contacts.

Malignant transformation *in vivo* or in cultured cells entails the loss of E-cadherin expression and the transition of differentiated (columnar) epithelial cells into a more elongated, mesenchymal phenotype, a process referred to as epithelial-to-mesenchymal transition (EMT) (6, 7). This phenotypic change includes loss of epithelial polarity, reduced E-cadherin-mediated cell-cell adhesion and up-regulation of mesenchymal markers

including N-cadherin, vimentin and fibronectin (FN), as well as matrix-degrading proteases (e.g. MMPs). In addition, increased expression and engagement of integrins causes activation of focal adhesion kinase (FAK), which in turn activates a PI3k/Akt survival pathway, and promotes cell migration and invasion (8, 9). These pro-metastatic functions are particularly important during extravasation and initial survival of single tumor cells in distant organ sites. Collectively, EMT promotes increased tumor cell migration, invasion into the stromal environment and anchorage-independent growth in culture (6, 7). It is therefore, not surprising that EMT has been linked to poor prognosis in many cancer types (10, 11). However, therapies that target specific signaling pathways that regulate EMT have so far proven disappointing in the clinic (12). The role of cadherins in Stat3 activation (3, 13), and FAK in Akt activation (8, 14), have previously been reviewed. In this mini-review, we summarize the prevailing evidence on the role of the cadherin/Stat3 and integrin/FAK pathways in cellular survival and metastasis.

Cell-cell adhesion – the cadherin/Stat3 pathway

The Stat3 transcription factor

Stat3 is activated by cytokine receptors especially of the IL6 family, receptor tyrosine kinases (RTKs) such as EGF, and non-receptor kinases such as Src. Stat3 is found to be overactive in human cancer and to be required for transformation by a number of oncogenes (15). Somatic Stat3 activating mutations have been recently reported in large granular lymphocytic leukemia (16) and inflammatory hepatocellular adenomas (17), while germline activating Stat3 mutations were reported to cause early onset autoimmune disease (18). However, persistent activation of Stat3 occurs mainly downstream of pro-oncogenic tyrosine kinases in the majority of cancer cases, and rather infrequently through a direct Stat3 mutation (17). Still, a constitutively active form of Stat3 (Stat3C) alone is sufficient to induce neoplastic transformation of cultured mouse fibroblasts (19), which points to an etiological role of Stat3 in neoplasia.

Stat3 and survival

In quiescent cells, Stat3 is inactive in the cytoplasm. Following receptor stimulation, Stat3 is phosphorylated at the crucial tyr-705 (Stat3-ptyr705) by Jak or Src family kinases, or the receptor itself (20). This is followed by Stat3 dimerization through reciprocal Src homology 2 (SH2)-ptyr interactions and translocation to the nucleus. The tyr-705 phosphorylated Stat3 dimer then binds a 9-bp sequence (TTCNNNGAA), thereby activating transcription of specific genes involved in cell division and survival, such as *myc, bcl-xL, mcl-1* and *survivin* (21). At the same time, Stat3 downregulates the tumor suppressor p53 (15, 22, 23), thereby suppressing apoptosis. Stat3 has also recently been shown to inhibit apoptosis by other mechanisms as well: activating the oxygen sensor HIF1α (hypoxia inducible factor–1α) transcription factor, which leads to increased aerobic glycolysis (24, 25); downregulating the mRNA's of mitochondrial genes, thereby reducing oxidative phosphorylation and ROS (reactive oxygen species) production (26, 27); enhancing, in a transcription-independent manner, the activity of ETC (electron transfer chain) complexes and glycolysis, through a tyr727-phosphorylation of Stat3, which localizes to the mitochondria rather than the nucleus (28); and, finally, by opposing (through tyr727 phosphorylation) the mitochondrial permeability transition pore (24, 29, 30). That is, Stat3, either through a direct effect upon the transcription of specific genes, or through an effect upon the mitochondria, offers a potent survival signal (Figure 1).

In addition to providing proliferation and survival signals, Stat3 contributes to cell invasion and metastasis (31, 32), through induction of angiogenesis by activating the genes for VEGF and HIF1α, enhancement of immune evasion by downregulating specific inflammatory chemokines and cytokines (21, 25, 33), and upregulation of matrix metalloproteinases (MMPs). Overall, as many pro-oncogenic signals converge on Stat3, it is unsurprising that elevated levels of Stat3 have been reported in nearly all human malignancies (34).

Cadherins in metastasis of prostate and breast cancer

Besides growth factors and oncogenes, engagement of cadherins, cell-cell adhesion molecules, as seen at confluence of cultured cells, was shown to induce a dramatic surge in Stat3-ptyr705 phosphorylation and activity in a number of cell lines [(2, 35–40), reviewed in (13)].

Cadherins are a superfamily of adhesive receptors that control the specificity, organization and dynamics of intercellular recognition and cell junction formation. These functions are crucial for the development, stability and homeostasis of tissue architecture and function

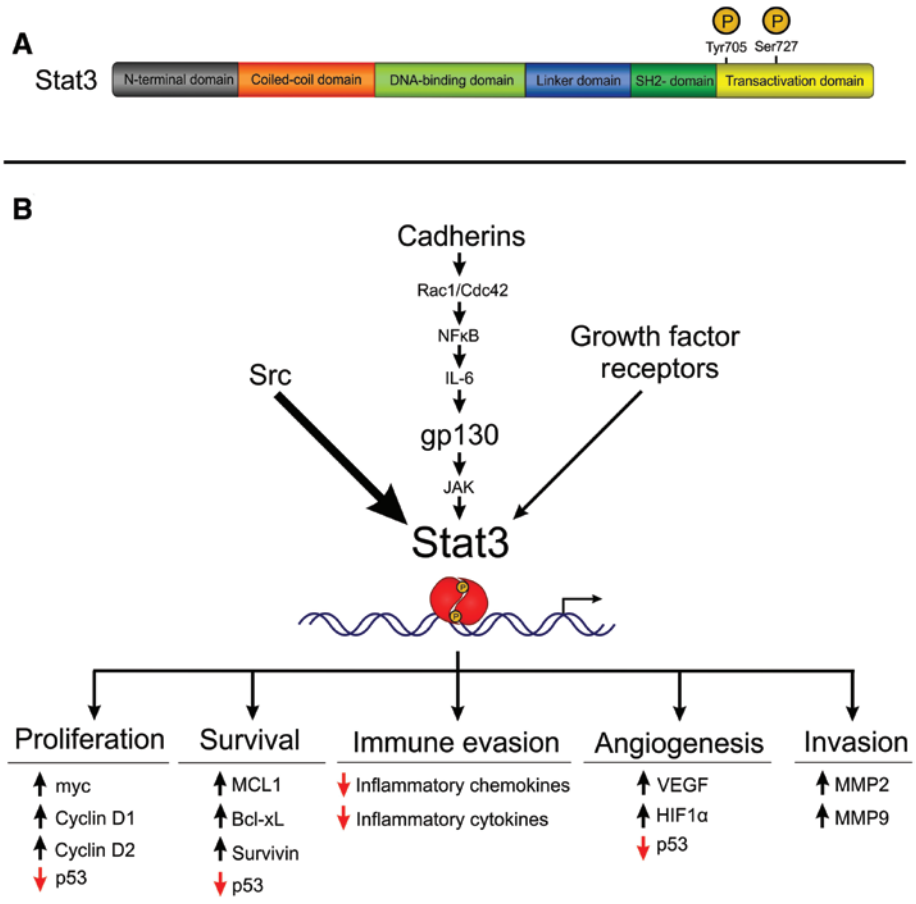

Figure 1: The Stat3 transcription factor.
(A) Stat3 functional domains. STAT family proteins (Stat1 to Stat6) differ within the transactivation domain at their C-terminus, but are otherwise similar in structure. The transactivation domain contains two phosphorylation sites, Ser-727 and Tyr-705 in Stat3. Following activation, tyrosine kinases, cytokine receptors or the associated Jak or Src family kinases (3, 21) mediate Stat3-ptyr705 phosphorylation. This permits dimerization through a reciprocal interaction between the phosphorylated residue and Stat3's SH2 domain, and the complex is then targeted to the nucleus. The Stat3, DNA binding domain, which recognizes a 9-bp consensus sequence (TTCNNNGAA), controls transcription by binding DNA in the promoter region of target genes. Ser727 phosphorylation is mediated by the Ras/Erk pathway and Stat3-pser727 is targeted to the mitochondria (160). At the same time, the coiled-coil domain contains a number of sites that facilitate protein-protein interactions and the N-terminal domain plays a role in stabilizing the Stat3 dimer (21). (B) Stat3 regulates a wide profile of cellular processes with its gene regulatory function. Stat3 controls a wide variety of cellular processes. It promotes cell proliferation by upregulating cell cycle genes such as myc, cyclin D1, cyclin D2 and $p21^{CIP/WAP}$ (79, 161) and downregulating the tumor suppressor p53 (23). Stat3 also contributes to cell survival by upregulating apoptosis inhibitors such as Bcl-xL, Mcl-1 and survivin (3, 22), by activating the oxygen sensor HIF1α (hypoxia inducible factor-1α) transcription factor, and by other transcriptional or non-transcriptional (not shown) mechanisms (24–26, 29, 30). In addition, Stat3 induces angiogenesis by activating the genes for VEGF and HIF1α, enhances immune evasion by downregulating inflammatory chemokines and cytokines (21, 25, 33), and, by upregulating matrix metalloproteinases (MMPs), Stat3 contributes to cell invasion and metastasis (31, 32). Due to these features, Stat3 contributes to a more aggressive cancer phenotype.

in all multicellular metazoans (41–43). Classical cadherins are glycoproteins that consist of an extracellular (EC) domain of five cadherin repeats (EC1 to EC5), a single transmembrane domain and a highly conserved intracellular region, which interacts with the cytoskeleton *via* the catenin family of proteins (44, 45). Upon calcium binding, the EC domains change conformation to form adhesive structures between adjacent cells, named adherens junctions (41, 46, 47). Such structures participate in cell polarity and differentiation, and provide local reinforcement against mechanical stress. Classical cadherins are subdivided into type I (E, N, M, P, R and C cadherin) and type II (VE- 6–12, 18–20, 22, 24) based on the structure of their EC domains (44).

The first discovered and best characterized classical type I cadherin is the E (epithelial)-cadherin, which is involved in the formation and maintenance of epithelial polarity (1, 42, 48). Early results showed that continued

plasma membrane expression of E-cadherin is required for cells to remain tightly associated within the epithelium, and it is currently accepted that the loss of E-cadherin not only contributes to a more aggressive cancer phenotype (by promoting metastasis and invasion), but is also a major initiating event in tumorigenesis (49, 50). This loss of E-cadherin can be induced by gene silencing leading to reduced expression, mutation, or aberrant localization of the mature protein, and has been shown to contribute to metastasis in many different types of human cancer such as pancreatic cancer, gastric cancer and breast cancer (43, 49). However, the loss of a cell's native cadherin is a prerequisite for metastasis in virtually all types of human cancer and is not an event limited to cancers of epithelial origin (49). Conversely, over-expression of E-cadherin in breast cancer cells inhibited metastasis to bone in a mouse model of bone metastasis (51, 52), demonstrating it has a pivotal role in maintaining the integrity of the native tissue.

Contrary to E-cadherin, the type II classical cadherin, cadherin-11, was shown to be actually required for prostate and breast cancer metastasis to the bone. In fact, cadherin-11 was originally isolated from the MC3T3-E1, mouse osteoblastic cell line. It is highly expressed in bone marrow stromal cells and osteoblasts (hence, OB- or osteoblast cadherin) (53) but it was later found to be constitutively expressed in a variety of normal tissues of mesodermal origin (54), as well as in cultured fibroblasts (35). It has long been noted that metastasis in prostate cancer patients occurs to bone with up to 80% frequency (55), with a high mortality as a result. Cadherin-11 is not expressed in normal human prostate epithelial cells but is frequently detected in prostate cancer, with its expression increasing from the primary tumor to metastatic disease to lymph nodes and bone. In fact, intracardiac injection of prostate cancer PC3 cells in a mouse tumor xenograft model leads to metastasis to the bone, which is significantly decreased upon cadherin-11 downregulation with specific shRNA expression, indicating that cadherin-11 is causally linked to bone metastasis (56). In fact, overexpression of cadherin-11 in PC3 prostate cancer cells was shown to increase their migration and invasion by increasing their interaction with osteoblasts through homophilic cadherin-11 binding (57). It was later demonstrated that inhibition of adhesion by an antibody specific for the EC3 domain of cadherin-11 (aa 343–348) thwarts the metastasis of PC3 cells to the bone (58). However, both prostate cancer cells and osteoblasts express N-cadherin as well, and a monoclonal antibody to N-cadherin also inhibited prostate cancer cell adhesion to fibronectin, as well as migration, invasion, metastasis and castration-resistance of prostate cancer cells implanted subcutaneously (59).

Regarding breast cancer, N-cadherin overexpression and engagement is reported to be associated with a highly invasive phenotype and motility in mammary cell lines (43, 60). In addition, high levels of cadherin-11 correlated with aggressiveness of breast cancer in lines cultured from breast cancers (61). Furthermore, a bone-metastatic, breast cancer cell line, MDA-MB-231-BO, had dramatically higher cadherin-11 levels than the parental one, or a brain-seeking line (62). Taken together, these results indicate that cadherin-11 and N-cadherin promote metastasis to the bone, in both prostate and breast cancer.

The metastasis-promoting ability of cadherin-11 and N-cadherin has been attributed to homophilic ligations of cadherins between tumor cells and osteoblasts. However, in other cancers, such as melanomas and head and neck cancers, hypermethylation-induced silencing of the cadherin-11 gene is actually a facilitating event for the scattering of tumor cells, by loosening cell-cell contacts and intravasation into blood and lymphatic vessels, with a reduction in metastasis as a result (63).

Cadherins activate Rho GTPases and Stat3

Cadherin engagement regulates the activity of the Rho family of small GTPases (64), which, in turn, regulates actin organization (65). Engagement of E-cadherin in HC11 mouse mammary epithelial cells induces a dramatic increase in total protein levels and activity of the small GTPases, Rac and Cdc42, through inhibition of proteasomal degradation (2, 66). Activated Rac leads to a surge in secretion of cytokines of the IL6 family through the transcription factor NFκB and Jak kinases, and this, in turn activates Stat3 in an autocrine manner [(2), reviewed in ref. (13)].

In addition to E-cadherin, recent results demonstrated that the mesenchymal cadherins, N-cadherin and cadherin-11, can also trigger a dramatic surge in Stat3 activity in mouse Balb/c3T3 fibroblasts by participating in a similar pathway [(35), reviewed in (3)]; this activation occurs through Rac and Cdc42 upregulation and transcriptional upregulation of IL6 family cytokines. Same as E-cadherin, the cadherin-11 mediated Stat3 activation is necessary for cell survival, proliferation and migration (35). A cadherin-induced activation of Rac was also observed for C- (67) and M-cadherin (68), strongly suggesting that this may be a feature common to all classical cadherins. Taken together, the above data indicate that the cadherin/Rac/IL6/Stat3 axis may be a pathway used by multiple cadherins of widely divergent functions. Moreover, the fact that the pro-metastatic cadherin-11 and

N-cadherin as well as the anti-metastatic epithelial E-cadherin, all activate Stat3 (43, 49, 56, 61), may point to Stat3 as a central survival, rather than metastasis factor. In fact, it has long been demonstrated that Stat3 inhibition in Src-transformed cells induces apoptosis, not simply reversion of the cell to a normal phenotype (69, 70).

Through Rb inhibition, a large variety of oncogenes increase the activity of the E2F family of transcription factors (the 'activating' E2F's, E2F1-3a), which are important cell cycle controllers (71). E2F activation leads to upregulation of genes not only involved in cell division (72), but also apoptosis-induction, through both p53-dependent and independent mechanisms [(73–76), reviewed in ref. (77)]. However, apoptosis is normally prevented, due to activation of Stat3 and/or PI3k by tyrosine kinase receptors induced by E2F, or directly by the oncogenes which had activated E2F in the first place, so that transformation does occur (Figure 2). Upon inhibition of Stat3 activity however, tumor cells (having high E2F levels) may succumb to apoptosis (39). Most importantly, inhibition of cadherin-11 or N-cadherin would induce apoptosis (through Stat3 inhibition) in metastatic tumor cells specifically, since normal cells, even cells with cadherin-11, would have low E2F activity hence would be spared [(3, 78) Raptis, Guy, Geletu in preparation]. It is interesting to note in this context that, as mentioned above, treatment with cadherin-11 specific antibodies following intracardiac injection of prostate cancer PC3 cells into mice caused tumor cell death (58). Based on our model, this could have been due to apoptosis triggered by the reduction in Stat3 activity, due to cadherin-11 inhibition (Figure 2).

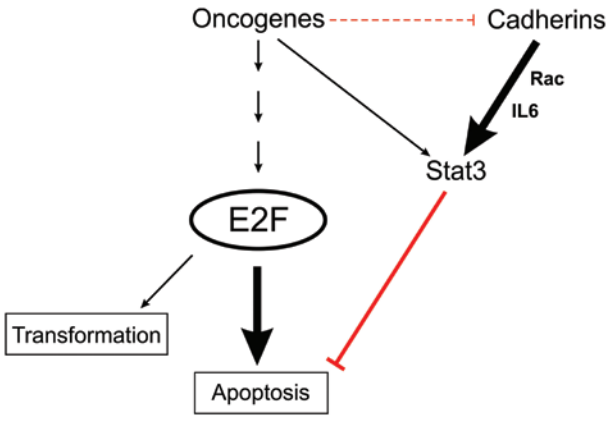

Figure 2: Model of Stat3 as a survival signal in cancer cells. Oncogenes such as activated growth factor receptors or Src activate the transcription factor family E2F1-3a, through the Ras/Raf/Mek/Erk1/2 pathway (77). E2F family proteins, in turn, can induce apoptosis through both p53-dependent and independent pathways. Opposing this effect, many oncogenes also activate Stat3 (and PI3k) which blocks apoptosis, so that transformation can occur. However, cadherin engagement may also be necessary to increase Stat3 activity further, even in cells expressing Stat3 activating oncogenes such as Src, and despite the fact that Src triggers the downregulation of cadherins, dependent upon its levels of expression (Guy, Raptis et al. manuscript in preparation).

In summary, the cadherin/Stat3 axis is emerging as a central survival pathway, which would be useful for tumor cells. Most importantly, in cases where cadherin-11 promotes metastasis, inhibition of cadherin-11 would promote apoptosis of metastatic cells specifically (through Stat3 inhibition), a finding which could have important therapeutic implications.

Stat3 inhibitors in clinical management of cancer

Stat3 activation has been described in almost 70% of both solid and hematological tumors (79, 80). Moreover, a variety of *in vitro* studies have shown that Stat3 inhibition reduces proliferation and survival of a wide variety of cancer cell lines with hyperactive Stat3, thus suggesting a reliance, i.e. an addiction, to this oncoprotein (81, 82). Interestingly, although genetic Stat3 inactivation causes embryonic lethality (83), many normal tissues are relatively unaffected by Stat3 loss (21, 84); this observation makes Stat3 an attractive target for cancer therapy. The search for Stat3 inhibitors led to platinum complexes such as CPA7 (85) and the peptide P^{p}YLKTK that targets the SH2 domain (86). However, despite their efficacy and specificity, the low cell permeation of phosphopeptides led to the search for smaller derivatives. This yielded molecules such as STA-21 (87), Stattic (88), S31–201 (89) and BP-1-102, a compound which is orally bioavailable (90). Despite their efficacy *in vitro* or in mouse xenografts *in vivo*, the above families of compounds have not yet reached the clinic.

A different class of inhibitors are the decoy oligodeoxynucleotides, first discussed over a decade ago (91), which associate with Stat3 in the cytoplasm and inhibit Stat3 DNA binding. Such a decoy, composed of a 15-bp duplex with free ends and phosphorothioate modifications of the 3′ and 5′ nucleotides, effectively inhibited cell proliferation in cancer cell lines isolated from patients with squamous cell carcinomas of the head and neck (HNSCC) by disrupting Stat3 activity (91), and inhibited tumor growth in xenografts *in vivo* (92). In a recent phase zero clinical trial, the decoy was injected into the tumors of HNSCC patients immediately prior to surgery and it was shown that expression of the Stat3 target genes, Cyclin D and Bcl-xL was reduced compared to control patients injected

with saline alone. Taken together, these data indicate that this oligonucleotide, while having limited toxicity, has a repressive effect on tumor gene expression when delivered intratumorally (93). Still, the decoy did not reduce tumor size in mouse xenografts when delivered intravenously, possibly due to its low stability. However, a modification linking the strands with hexaethylene glycol spacers to render it cyclical improved its stability and its ability to inhibit tumor growth in xenografts (93).

Another small molecule inhibitor of Otsuka Pharmaceutical Co, OPB-31121, is the first selective Stat3 inhibitor in phase I clinical trials involving patients with hematopoietic tumors (85) as well as some primary solid tumors (94). Preclinical studies demonstrated promise of OPB-31121 as a therapeutic agent where it showed a strong growth inhibitory effect in a wide variety of malignant hematopoietic cell lines *in vitro* (95). In addition, OPB-31121 increased survival and reduced the number of transplanted human leukemia cells in an *in vivo* mouse model, but did not affect the growth of healthy cord blood cells (95). But thus far, no effect on metastasis has been determined. Nevertheless, these data suggest that the inhibitory effect of OPB-31121 is highly selective to tumor cell Stat3 activity. Two phase I clinical trials have been completed (NCT00955812/NCT00511082) and currently a phase I/II clinical trial is underway, but the results have not been reported as yet. Recently, the IL-6/Jak2/Stat3 pathway has been shown to be preferentially activated in basal-like breast cancer cells, and that inhibition of Jak2 with NBP-BSK805 blocks growth of patient-derived breast tumor xenografts (96).

In conclusion, while transcription factors such as Stat3 had been widely regarded previously as un-druggable targets, it is becoming clear that specific inhibitors of Stat3 or its upstream activator Jak2 may have a prominent place in the clinic (97, 98), especially in combination with drugs that target complementary survival pathways such as the integrin/FAK signaling network (described below).

Cell adhesion to the extracellular matrix (ECM) – the integrin/FAK pathway

Role of integrins in EMT

Integrins are heterodimeric transmembrane receptor proteins which mediate ECM-cell adhesion, and are important in maintaining normal epithelial polarity and function (4). β1-integrin forms heterodimers with α1, α2, α3, α5 and α6 chains, which can bind to various ECM proteins (4). Of particular importance in the normal mammary gland is the α2β1 and its ligands, collagens I and laminin V. Engagement of β1-integrin oligomers promotes expression of the metalloproteinase, MMP14, required in the branching morphogenesis of the developing mammary end bud, and regulates transcription and protein secretion of β-casein during lactation (99). In addition, α6β4-integrin heterodimers form hemi-desmosomes, which are cell adhesion complexes that anchor the intermediate filament cytoskeletal network of myoepithelial cells to underlying basement membrane proteins (5).

Aberrant signaling during oncogenesis can lead to increased expression of β1family integrins (e.g. α2β1 and α5β1), which promote increased cell-ECM adhesion, migration and invasion, characteristic of the EMT phenotype (100). β1 integrin expression is also predictive of poor patient survival in many cancer types including breast (101, 102). The important role of integrins in malignancy has been demonstrated by our group and others: Weaver et al. (103) demonstrated that antibody-mediated inhibition of β1 integrin causes reversal of the transformed phenotype of cancer cells in 3-D Matrigel cultures, while we showed that antibody-mediated β1 integrin block attenuates pulmonary metastasis *in vivo* (104). In addition, targeted disruption of β1-integrin abrogates polyoma middle T (PyMT)-mediated mammary tumorigenesis in a transgenic mouse model (105). Similarly, shRNA knockdown of β4 integrin blocks osteosarcoma metastasis (106), and targeted ablation of β4 integrin blocks ErB2-induced mammary tumorigenesis (107).

Focal adhesions (FAs) and focal adhesion kinase (FAK)

FAs are the main sites of attachment of cells to the ECM. FAs mediate the activation of downstream signaling pathways important for cytoskeletal reorganization and the generation of traction forces during cell migration (108). Indeed, highly motile tumor cells require FA formation and are capable of promoting efficient FA turnover. Failure of FAs to disassemble properly causes an increase in the number and size of FAs, therefore, leading to increased cell adhesion and reduced migration (109, 110). The cytoskeletal protein FAK binds to β1 integrins following adhesion to ECM substratum, thereby initiating formation of FAs. A critical step in FA disassembly is the cleavage of FAK (as well as other FA proteins) by the cysteine protease calpain (109, 111). Our group has also shown that the cytoskeleton

cross-linker protein, Ezrin, is required for maintaining proper localization and activity of calpain in this process (110). Interestingly however, studies on the regulation of FA dynamics by FAK have shown that FAK-deficiency does not abrogate FA assembly, but rather leads to abnormal FA disassembly. This results in cells that are more 'sticky' and do not migrate or invade, indicating that the role of FAK in FAs is complex (111).

Src/FAK signaling

FAK is a major substrate of the non-receptor tyrosine kinase Src, and is a key regulator of EMT, a process dependent on integrin signaling (112). Src is over-expressed and activated in many invasive cancers (113), and is required for mammary tumorigenesis in PyMT-transgenic mice (114–116) as well as for ErB-2-mediated transformation (116). However, although an activating mutation (Y527F, lacking the negative regulatory site) of the avian Src homologue can induce mammary hyperplasia, it is insufficient for tumorigenesis in transgenic mouse models (117). On the other hand, a truncating mutation in Src from codon 531 in the 3′-region of the Src gene encompassing the negative regulatory site, has been detected in metastatic lesions in human colorectal cancer, but not in non-metastasizing tumors (117, 118). Taken together, these data indicate that additional signaling events are required, either for Src-induced transformation or metastasis.

FAK binds to the β1 integrin cytoplasmic domain and the SH2 domain of Src, thereby sequestering Src in FAs (118, 119). In fact, an increase in FAK protein levels has also been shown in multiple cancer types, including breast, and is frequently associated with a more malignant phenotype (120–122). The Src/FAK protein-protein complex is required for Src-triggered signaling events that drive EMT and tumor progression. Interestingly, the Src/FAK complex can activate specific RTKs such as EGFR (123, 124) and Met (125). In this way, integrin signaling, through Src and FAK, provides a ligand-independent pathway for RTK activation during tumorigenesis.

Specific structural domains of FAK regulate its auto-activation, and the activation of downstream effectors through protein-protein interactions (Figure 3). The FERM (4.1 protein, Ezrin, Radixin, Moesin) homology domain of FAK mediates interactions with β1-integrins, as well as the cytoskeletal protein Ezrin and RTKs including EGFR and PDGFR (8, 126). These interactions force FAK into an open conformation, thereby allowing the auto-phosphorylation of tyrosine 397 in the kinase domain of FAK [reviewed in (127)]. Src then binds to the phosphorylated tyrosine 397 through its SH2 domain and subsequently phosphorylates additional tyrosine residues, 576 and 577, in the FAK kinase domain, thus further increasing the activity of FAK (127). Src can also phosphorylate tyrosines 861 and 925 on FAK. Finally, the Focal Adhesion Targeting (FAT) domain of FAK associates with paxillin and talin, thereby recruiting FAK to the focal adhesion complex.

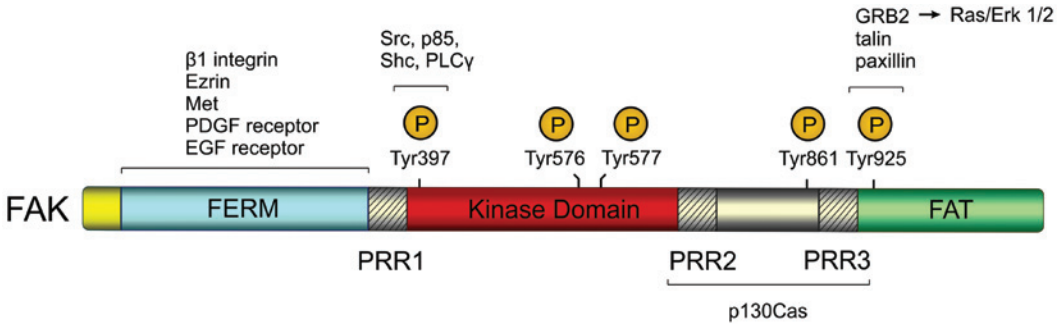

Figure 3: Domain structure and phosphorylation sites of FAK.
FAK consists of a FERM (4.1 protein, Ezrin, Radixin, Moesin) homology domain, three Proline-rich regions (PRR1, PRR2, PRR3), a kinase domain, and a focal adhesion targeting (FAT) domain. The FERM domain interacts with β1-integrins, the cytoskeletal protein ezrin, and RTKs including Met and EGF receptor. These interactions force FAK into an open conformation, thereby allowing the auto-phosphorylation of tyrosine 397 in the kinase domain of FAK. Src then binds to the phosphorylated tyrosine 397 through its SH2 domain and subsequently phosphorylates additional tyrosine residues, 576 and 577 in the FAK kinase domain, thus further increasing the activity of FAK. Src also phosphorylates tyrosines 861 and 925 on FAK. FAK-ptyr397 can also bind and activate other SH2 containing proteins including p85 subunit of PI3k, PLCγ and Shc. FAK-ptyr861 acts through intramolecular interactions to promote increased binding of proteins with Src-homology-3 (SH3) domains such as p130cas to the proline rich domains PR2 and PR3 of FAK. FAK-ptyr925 is involved in Erk1/2 activation through GRB2 binding and is located in the FAT domain that targets FAK to focal adhesions. The FAT domain associates with paxillin and talin, thereby recruiting FAK to the focal adhesion complex. [Adapted from (14).]

Downstream effectors of Src/FAK

The Src/FAK regulatory network activates downstream effectors that promote tumor formation and metastasis [reviewed in (14)]. Binding of the p85 subunit of PI3k to FAK-ptyr397 activates the PI3k/Akt survival pathway. In addition, recruitment of Grb2 to FAK ptyr925 leads to activation of the Ras/Mek/Erk1/2 pathway, which in turn stimulates cell cycle progression through **c**yclin D1 upregulation and decreased p21 expression [reviewed in (8)]. Erk1/2 also upregulates the Bcl-xL/Bcl2-dependent cell survival pathway through the cAMP response element-binding (CREB) protein, with concomitant down-regulation of the pro-apoptotic proteins Bim and Bad through ubiquitination and degradation (9, 128). FAK-ptyr861 acts through intramolecular interactions to promote increased binding of proteins with Src-homology-3 (SH3) domains such as p130cas to the proline rich domains PR2 and PR3 of FAK (14). Activation of p130cas and c-Jun N-terminal kinase (JNK) induces expression of MMPs required for cell migration and invasion (128). Collectively, these signaling events underscore the importance of integrin-based cell matrix adhesion and the Src/FAK regulatory network as a pro-metastasis pathway and potential target for anti-cancer therapy.

FAK promotes resistance to Anoikis

Anoikis is a process that induces death of normal cells in response to detachment from the ECM substratum (129). E-cadherin-dependent cell-cell contacts play a critical role in protecting differentiated epithelia from cell death, through activation of Stat3-dependent pro-survival pathways as described above. In fact, loss of both ECM attachment and intercellular junctional complexes in epithelial tissues leads to rounding up and shedding of single epithelial cells which are highly susceptible to anoikis. Cell death occurs through ligand binding to members of the tumor necrosis factor receptor (TNFR) superfamily of death receptors, including TNFR, TNF-related apoptosis inducing ligand (TRAIL) and the Fas-associated death domain (FADD) protein. These pathways lead to the activation of the caspase cascade that mediates substrate proteolysis leading to activation of pro-apoptotic (e.g. Bid and Bim), or inactivation of pro-survival (e.g. Bax and Bak), proteins. The details of these pathways have been described elsewhere (9, 129), and are beyond the scope of this review.

During EMT, the E-box binding transcription factors, Snail, Slug and Twist, act as transcriptional repressors of genes that encode proteins that form junctional proteins such as cadherins, desmosomes and tight junctions, and thereby suppress cell-cell contacts and downstream survival signals. Conversely, EMT induces expression of β1 family integrins, resulting in increased engagement of integrin-based signaling which is critical in sustaining a pro-survival response through the FAK signaling network described above (100). Moreover, FAK has emerged as a key driver of tumorigenesis, as it is linked to survival and maintenance of cancer stem cells, as well as anchorage independent growth and dissemination of tumor cells (130).

Anchorage-independent growth and dissemination of tumor cells

During malignant transformation, tumor cells acquire the ability to grow under anchorage-independent conditions such as in soft agar cultures. Under these conditions, v-Src or the activating avian Src mutant (Y527F) still require integrin engagement for activation of FAK and its downstream effectors (112, 113). Interestingly however, survival signaling is provided through increased expression of β1-integrins during EMT as described above, causing integrin clustering at the surface of detached tumor cells (131). These surface patches of integrin clustering are sufficient to promote auto-phosphorylation of FAK at Y397 and phosphorylation of FAK Y577 by c-Src, which together lead to increased cell survival as well as colony growth (125, 132). Under these conditions, the formation of fibronectin fibrils at the outer edge of FA complexes is required for appropriate integrin signaling, as shown by the fact that an inhibitory molecular fragment of FN (70 kDa) blocked FAK Y397 phosphorylation and anchorage-independent growth (133, 134). Thus, tumor cells have the capacity to modify their juxtamembrane ECM-integrin composition to sustain strong integrin signaling in detached cells.

Recent studies have identified signaling events leading to FAK activation completely independent of cell matrix adhesion, that can promote tumor progression. One such mechanism is linked to Ezrin, which is over-expressed in many invasive cancer types and is associated with poor patient survival (135, 136). Poullet and colleagues have shown that over-expression of Ezrin, which binds via its FERM domain to FAK in epithelial cells, promotes autophosphorylation of FAK at Y397 independently of integrin engagement (126). This event allows activation of the PI3k/Akt survival pathway by FAK, under non-adherent conditions. Furthermore, ezrin itself can activate the PI3k/Akt pathway (137). Collectively, these pathways

can promote survival and growth of single disseminating tumor cells that have no engagement of cell-cell (cadherin) or ECM-cell (integrin)-dependent adhesions. These studies further implicate FAK and ezrin as key drivers of tumor progression and metastasis.

Interestingly however, disseminating tumor cells that are able to maintain cell-cell contacts [and therefore, circulate as tumor emboli (138)], exhibit greater organ colonization potential when compared to single tumor cells (139), suggesting a survival advantage to tumor cells (i.e. through the cadherin-Stat3 survival pathway) that metastasize in a collective manner. As the majority of disseminated tumor cells die within 24 h of initial organ seeding, known as metastatic inefficiency (140), survival signaling becomes crucial once again, for the ability of tumor cells to establish metastatic lesions.

The FAK signaling network in metastatic cancer

Increased FAK expression in many cancers, including breast, gastric, and laryngeal carcinomas, is frequently associated with amplification of the protein tyrosine kinase 2 (PTK2) gene, which encodes FAK (120, 141). Furthermore, FAK mRNA expression is negatively regulated by p53, and is frequently increased in tumors that express mutated (inactive) p53. In addition, increased phosphorylation of FAK at Y397 (which recruits Src and the p85 subunit of PI3K to FAs) was found in invasive ovarian tumors, but not in their normal epithelial counterparts (142). Phosphorylation at Y925 FAK (a Src substrate) is also upregulated and predicts poor survival in colorectal cancer (143).

A variety of studies using animal tumor models have demonstrated a causal role for FAK in tumorigenesis and metastasis (141) which showed that conditional deletion of FAK abrogated tumor outgrowth and metastasis in a transgenic mouse model of PyMT-induced mammary tumorigenesis. Furthermore, siRNA depletion of FAK induced apoptosis and abrogated metastasis of human breast cancer cell lines carrying oncogenic mutations of Ras or PI3k (141). This effect was mimicked by knockdown of p130cas, indicating that FAK supports tumor progression through p130cas in these cell lines. However, this may not always be the case, as depletion of FAK, or expression of a dominant negative (C-terminal FRNK) domain, had minimal effects on survival and proliferation of established breast cancer cell lines under adherent conditions (128). Together, these findings underscore a dominant survival role of FAK in tumorigenesis and metastasis.

In addition to the pro-metastatic effects of FAK described above, FAK has also been shown to be important in the peri-tumoral stromal microenvironment. Recent studies with conditional FAK knockout mice have demonstrated a regulatory role of endothelial cell (EC) FAK in vascular development and tumor angiogenesis [reviewed in (120)]. FAK also regulates directional migration and invasion of cells of the myeloid and lymphoid cell lineages that mediate inflammatory responses in cancer and other diseases (120, 143). Thus FAK regulates both intrinsic (tumor) and extrinsic (stroma) components of the tumor microenvironment, making this cytoskeletal protein into an attractive treatment target.

FAK inhibitors in clinical management of cancer

A strong rationale for targeting FAK in the treatment of breast cancer is its broad protein-protein interactome, allowing it to regulate multiple signaling pathways that are positively involved in tumor progression. Moreover, increased FAK expression in ovarian and colorectal cancers has been shown to predict disease relapse in cancer patients treated with the cytotoxic chemotherapeutic agents, doxorubicin and paclitaxel (121, 144), suggesting that increased FAK signaling promotes resistance of tumors to these cytotoxic drugs. Furthermore, a similar role of Src/FAK signaling has been proposed in acquired resistance of HER2-positive cancers following Trastuzumab treatment (145), and high EGFR expressing cancers following treatment with Erlotinib (146). In support of this notion, our group has shown increased phosphorylation of FAK-tyr925 in MDA-MB-231 tumor xenografts expressing an activated Src mutant following treatment with the Src inhibitor Dasatinib in a neoadjuvant setting (Carefoot, Elliott and Raptis, in preparation). Interestingly, while the rate of primary tumor growth was significantly reduced, pulmonary metastasis was not attenuated. This finding suggests that increased activation of the integrin/FAK pathway is linked to residual tumor outgrowth and metastasis, and may help to explain why clinical trials involving dasatinib as a single agent have yielded disappointing results (147, 148). That is, upregulation of the integrin/FAK signaling pathway may contribute to inherent or acquired resistance to specific targeted therapies including dasatinib, and the frequent failure of their use in the treatment of solid tumors (144, 146, 148)

A number of small molecule ATP-competitive inhibitors of FAK targeting the Y397 autophosphorylation site

have been validated as anti-metastatic agents in tumor xenograft models (149, 150). Recently, FAK inhibitors have shown marked synergistic apoptotic effects in combination with the doxorubicin (150) and paclitaxel (144) in preclinical cell-line based models. In addition, combined therapy with a FAK inhibitor (PF-562,271) and a multi-targeted RTK inhibitor (Sunitinib) has been shown to markedly increase apoptosis *in vitro*, and effectively block growth and metastasis of human hepatocellular carcinoma cells in rat xenografts compared to each agent alone (151). Similarly, combined treatment with the FAK inhibitor Y15 and the Src inhibitor PP2 decreased human colon carcinoma growth and enhanced the efficacy of 5-fluorouracil chemotherapy in nude mouse xenografts (150) (Figure 4).

Some FAK inhibitors are currently being used in Phase I and II clinical trials with promising results (120). Notably, a Pfizer FAK inhibitor, PF-00562271, showed disease-stabilizing effects in some ovarian, colorectal, and bile duct tumor patients (152). A future approach would be to test for efficacy of approved inhibitors that target both Src/FAK and Stat3 in cancer patients (153, 154), as targeting multiple nodes in growth/survival pathways has been used successfully in preclinical models. For example, the pan-CDK inhibitor flavopiridol was found to synergize with the Raf inhibitor sorafinib in a preclinical xenograft model of triple negative breast cancer (155), and even more pronounced synergy has been observed with the combination of Raf with Mek inhibitors in this same model (P. Greer, personal communication). In further

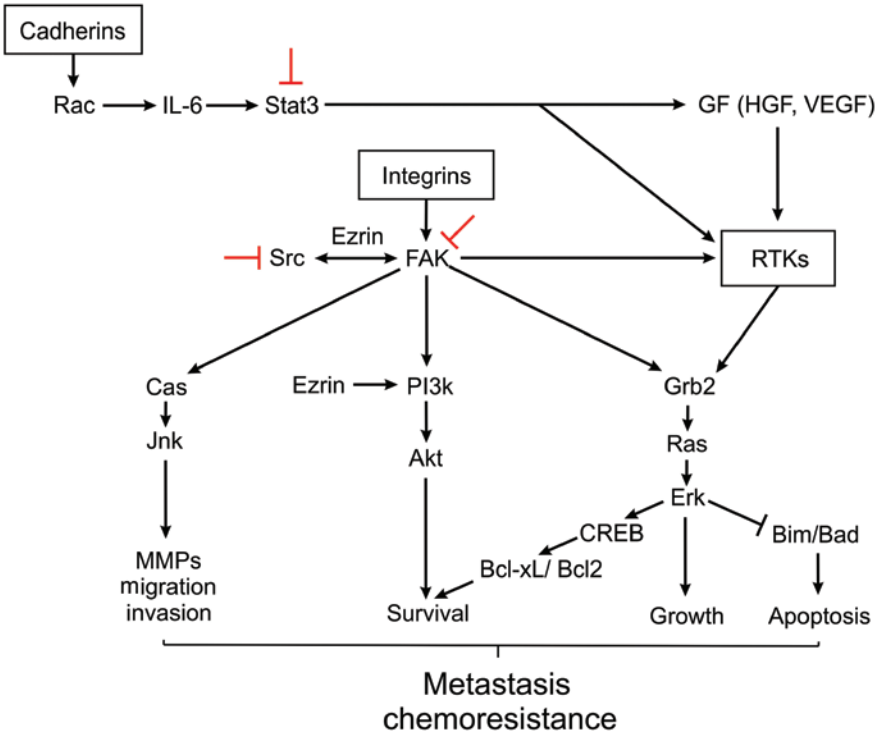

Figure 4: The Cadherin/Stat3-Integrin/Fak signaling axis in cancer metastasis.
Both cell-cell and cell-matrix adhesions are important for tumor formation and metastasis. Classical cadherins (E, N, 11) mediate cell to cell interactions and activate Stat3 through Rac/IL-6, thereby upregulating cellular survival [reviewed in (14)]. Stat3 can also activate transcription of growth factors HGF and VEGF, as well as the RTK Met (162). Cell-ECM adhesion is mediated through integrin receptors that bind to the FAK/Src complex (120). Binding of the p85 subunit of PI3k to FAK-ptyr397 activates the PI3k/Akt survival pathway. Ezrin also promotes activation of the Src/FAK complex, and can activate the PI3k/Akt axis and survival independent of FAK (137). In addition, recruitment of Grb2 to FAK-ptyr925 leads to activation of the Ras/MEK/Erk1/2 pathway, which in turn stimulates cell cycle progression through cyclin D1 upregulation and decreased p21 expression. ERK1/2 also upregulates the Bcl-xL/Bcl2-dependent cell survival pathway through the cAMP response element-binding (CREB) protein, with concomitant down regulation of pro-apoptotic proteins Bim and Bad through ubiquitination and degradation. FAK-ptyrY861 promotes increased binding of p130cas to the proline rich domains PR2 and PR3 of FAK. Activation of p130cas and c-Jun N-terminal kinase (JNK) induces expression of MMPs required for cell migration and invasion. Interestingly, despite the fact that mutationally activated Src is a potent Stat3 activator, the integrin/FAK/Src signal is insufficient to activate Stat3. Red markers indicate potential drug-target strategies. Collectively, these signaling events underscore the importance of Cadherin/Stat3 and Integrin/FAK signaling as pro-metastasis and chemo-resistance pathways and potential targets for anti-cancer therapy.

support of this strategy, a clinical trial involving combination treatment of a FAK inhibitor GSK2256098 with a Mek inhibitor, trametinib, has shown promise in the treatment of solid tumors (156).

Conclusions and future directions

Cell survival is a prerequisite for any tissue function. In cultured, non-transformed cells grown to low densities the integrin/FAK signal activates PI3k/Akt and survival, in the absence of cadherin engagement. Interestingly, the integrin/FAK signal does *not* activate Stat3, and is not required for cadherin-dependent Stat3 activation in non-transformed cells (Niit, Elliott, Raptis, manuscript in preparation). The endogenous integrin/FAK/Src signal is therefore, insufficient to activate Stat3, despite the fact that mutationally activated Src is a potent Stat3 activator (69, 70). Additional mechanisms, involving Jak and/or activated receptors acting in a scaffolding role (20) may also be required for Stat3 activation.

On the other hand, upon cadherin engagement at high cell densities, activation of both the cadherin/Stat3/survivin and integrin/FAK/Akt pathways can promote survival. Stat3 can then activate transcription of specific genes such as VEGF (33) and HGF (157), and stimulate an HGF/Met autocrine loop in breast and other invasive cancer types (158). That is, co-ordinate activation of these complementary pathways could greatly enhance survival and growth of disseminated tumor cells at distant metastastic sites, and would be expected to promote resistance to chemotherapeutic agents. Furthermore, increased Src/FAK activation could compensate for decreased cadherin/Stat3 signaling in tumors treated with Stat3 inhibitors, and vice versa, resulting in outgrowth of tumor variants resistant to the respective targeted anti-cancer therapies. In this scenario, combination treatment with inhibitory drugs targeted against Src/FAK and Stat3 would be expected to enhance patient survival (159).

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List of abbreviations

Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	bcl-2-like protein 4
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Bim	Bcl-2-like protein 11
Cdc42	cell division control protein 42
CREB	cAMP response element-binding protein
E-cadherin	Epithelial cadherin
E2F	E2 factor
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ErB2	erythroblastosis protein-2
Erk 1/2	extracellular signal regulated kinases 1/2
ETC	electron transport chain
FA(s)	focal adhesion(s)
FADD	Fas-associated death domain protein
FAK	focal adhesion kinase
Fas	FAS cell surface death receptor
FAT	focal adhesion kinase targeting domain
FERM	four point one Ezrin Radixin Moesin binding domain
FN	fibronectin
Grb2	growth factor receptor-bound protein 2
HER2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HIF1α	hypoxia inducible factor-1α
HNSCC	head and neck squamous cell carcinoma
IL-6	interleukin 6
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
M-cadherin	myotubule cadherin
Mek	mitogen-activated protein kinase kinase
Met	hepatocyte growth factor receptor (HGFR)

Mcl-1	myeloid cell leukemia 1
MMP(s)	matrix metalloproteinase(s)
N-cadherin	neuronal cadherin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OB-cadherin	osteoblast cadherin (cadherin-11)
p130cas	breast cancer anti-estrogen resistance protein 1 (BCAR1)
P-cadherin	placental cadherin
PDGFR	platelet-derived growth factor receptor
PI3k	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKB	protein kinase B (Akt)
PLCγ	phospholipase C-gamma
PTK2	protein tyrosine kinase 2
PyMT	polyoma virus middle T antigen
Rac	Ras-related C3 botulinum toxin substrate
Raf	rat fibrosarcoma
Ras	rat sarcoma
Rb	retinoblastoma protein
ROS	reactive oxygen species
RTK(s)	receptor tyrosine kinase(s)
SH2	Src homology 2 domain
SH3	Src homology 3 domain
Shc	Src homology 2 domain containing transforming protein 1
shRNA	short hairpin RNA
siRNA	small interfering RNA
Slug	zinc finger protein SNAI2
Snail	zinc finger protein SNAI1
Stat3	signal transducer and activator of transcription-3
Stat3C	constitutively active signal transducer and activator of transcription-3
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand
VEGF	vascular endothelial growth factor

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