The Cain and Abl of Epithelial-Mesenchymal Transition and Transforming Growth Factor- β in Mammary Epithelial Cells

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Key Words

Breast cancer • c-Abl • Epithelial-mesenchymal transition • Metastasis • Signal transduction • Transforming growth factor- β

Abstract

Transforming growth factor- β (TGF- β) normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, by inducing MEC apoptosis, and by creating cell microenvironments that maintain MEC homeostasis and prevent their uncontrolled growth and motility. Mammary tumorigenesis elicits dramatic alterations in MEC architecture and microenvironment integrity, which collectively counteract the tumor-suppressing activities of TGF- β and enable its stimulation of breast cancer invasion and metastasis. How malignant MECs overcome the cytostatic actions imposed by normal microenvironments and TGF- β , and how abnormal microenvironments conspire with TGF- β to stimulate the development and progression of mammary tumors remains largely undefined. These knowledge gaps have prevented science and medicine from implementing treatments effective in simultaneously targeting abnormal cellular microenvironments, and in antagonizing the oncogenic activities of TGF- β in developing and progressing breast cancers. c-Abl is a ubiquitously expressed nonreceptor protein tyrosine kinase that essentially over-

sees all aspects of cell physiology, including the regulation of cell proliferation, migration and adhesion, as well as that of cell survival. Thus, the biological functions of c-Abl are highly reminiscent of those attributed to TGF- β , including the ability to function as either a suppressor or promoter of tumorigenesis. Interestingly, while dysregulated Abl activity clearly promotes tumorigenesis in hematopoietic cells, an analogous role for c-Abl in regulating solid tumor development, including those of the breast, remains controversial. Here, we review the functions of c-Abl in regulating breast cancer development and progression, and in alleviating the oncogenic activities of TGF- β and its stimulation of epithelial-mesenchymal transition during mammary tumorigenesis.

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Abbreviations used in this paper

BCR	break-point cluster region
CML	chronic myelogenous leukemia
CST-Abl	constitutively active c-Abl
EMT	epithelial-mesenchymal transition
MEC	mammary epithelial cell
PTK	protein tyrosine kinase
TGF- β	transforming growth factor- β

Introduction

Transforming growth factor- β (TGF- β) is a ubiquitous cytokine that fulfills fundamental roles during embryonic development, cellular differentiation, wound healing and tissue remodeling, as well as immune homeostasis [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. In addition, TGF- β also plays an essential function in maintaining normal epithelial cell and tissue architecture, a regulatory mechanism that becomes disrupted in developing neoplasms. Indeed, as neoplastic lesions progress and become invasive, they typically circumvent the tumor-suppressing activities of TGF- β and paradoxically convert this cytokine into a potent promoter of metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Pardali and Moustakas, 2007; Barcellos-Hoff and Akhurst, 2009; Wendt et al., 2009a]. Recent evidence has established epithelial-mesenchymal transition (EMT) as being a vital component involved in initiating oncogenic TGF- β signaling in normal and malignant cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Indeed, TGF- β is a master regulator of EMT and its ability to engender polarized epithelial cells to (1) downregulate their expression of genes associated with epithelial phenotypes, including those operant in forming adherens and tight junctions; (2) remodel their actin cytoskeletons and microtubule networks; and (3) upregulate their expression of genes associated with mesenchymal phenotypes and cell motility [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. The process of EMT has recently been categorized into 3 distinct biological subtypes [Kalluri and Weinberg, 2009], namely type 1 (embryonic and developmental EMT), type 2 (tissue regeneration and fibrotic EMT) and type 3 (cancer progression and metastatic EMT). The linkage of type 3 EMT to the development of metastasis and poor clinical outcomes [Thiery, 2003] has led to intense research efforts aimed at developing novel chemotherapeutics capable of inhibiting oncogenic EMT, and as such, of improving the clinical course of patients with metastatic disease. Alternatively, identifying the molecular mechanisms that promote mesenchymal-epithelial transition (MET), which phenotypically and morphologically reverses the activities of EMT, may also offer new inroads to impede or thwart primary tumor metastasis, an idea echoed by those who attended the 3rd International TEMTIA meeting that was held in Krakow, Poland, in 2007.

c-Abl is a multifunctional nonreceptor protein tyrosine kinase (PTK) that localizes to the plasma membrane, cytoplasm and nucleus where it governs a variety of cel-

lular functions and activities, including the (1) transduction of integrins and growth factor receptor signals; (2) induction of cell cycle arrest initiated by DNA damage; (3) regulation of actin cytoskeletal dynamics; and (4) interaction with numerous adaptor proteins and scaffold complexes [Pendergast, 1996; Plattner et al., 1999; Hamer et al., 2001; Woodring et al., 2001; Pendergast, 2002; Woodring et al., 2002; Zandy and Pendergast, 2008]. In addition, c-Abl and its relative Arg are unique among nonreceptor PTKs in that both molecules house direct actin-binding domains that enable c-Abl to sense and respond to extracellular signals coupled to altered actin cytoskeletal architectures [Woodring et al., 2001, 2002; Zandy and Pendergast, 2008]. It is interesting to note that the diverse and complex biological functions of c-Abl are surprisingly reminiscent of the pathophysiological actions of TGF- β , including its dichotomous behavior exhibited during tumorigenesis. For instance, the tumor-promoting activities of c-Abl are best exemplified by its causal initiation of chronic myelogenous leukemia (CML), wherein c-Abl is translocated and fused to the break-point cluster region (BCR) on chromosome 22, resulting in the generation of a constitutively active Abl kinase fusion protein [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008]. Moreover, the pharmacological development and clinical implementation of imatinib (also known as Gleevec or STI-571), which targets the ATP-binding site in the c-Abl kinase domain and inhibits its phosphotransferase activity [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008], has significantly improved the treatment of CML and served as a model for the rationale design of protein kinase inhibitors [Druker, 2006; Soverini et al., 2008]. Although dysregulated c-Abl activity clearly promotes tumorigenesis in hematopoietic cells, the role of c-Abl in regulating tumorigenesis in solid tumors remains controversial. In fact, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression failed to observe any clinical benefit in imatinib-treated breast cancer patients. Moreover, these same studies found imatinib to cause significant toxicity and elicit disease progression in breast cancer patients [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Along these lines, our recently published study showed that imatinib administration failed to provide any therapeutic benefit to mice bearing aggressive mammary tumors, and instead, this same pharmacological treatment tended to produce larger breast tumors as compared with those observed in their vehicle-treated counterparts (fig. 1) [Allington et al., 2009]. Remarkably,

this same study demonstrated that engineering late-stage metastatic breast cancer cells to stably express a constitutively active c-Abl mutant resulted in their morphologic and phenotypic reversion both *in vitro* and *in vivo*, as well as circumvented the oncogenic activities of TGF- β and its ability to induce EMT [Allington et al., 2009]. Clearly, a deeper and more thorough understanding of pathophysiological functions of c-Abl in regulating solid tumor development and progression is needed for science and medicine to successfully advance the use of targeted chemotherapies against c-Abl and against various effectors activated by oncogenic TGF- β signaling.

Here, we review recent findings that directly impact our understanding of the dichotomous roles played by c-Abl during mammary tumorigenesis and discuss emerging evidence suggesting that chemotherapeutic targeting of c-Abl activation, not inhibition, may offer new inroads to suppress breast cancer progression and the oncogenic activities of TGF- β , particularly its induction of EMT and metastasis in developing neoplasms of the breast.

Abl Signaling and Cancer Progression

c-Abl was originally identified as the cellular counterpart to the Abelson murine leukemia virus, v-Abl [Wang et al., 1984]. Subsequent studies established that c-Abl exists in 2 isoforms in mammals (1a or 1b in humans and I or IV in mice), and that this large (approximately 140 kDa) nonreceptor PTK contains a variety of modular domains that enable c-Abl to bind numerous signaling and scaffolding proteins. Figure 2 depicts the structural features of human and murine c-Abl isoforms, both of which house (1) Src homology 2 (SH2) and SH3 domains; (2) a proline-rich adaptor-binding motif; (3) a PTK domain; (4) 3 nuclear localization signals and 1 nuclear export signal; (5) 3 high-mobility group-like boxes that function cooperatively in binding DNA; and (6) globular and filamentous actin-binding domains [Woodring et al., 2002; Hunter, 2007]. The presence of functional nuclear localization signals and nuclear export signal motifs localizes c-Abl to both the cytoplasmic and nuclear compartments in quiescent cells, as well as enables c-Abl to translocate to the nucleus in response to a variety of extracellular stimuli [Lewis et al., 1996; Wen et al., 1996; Taagepera et al., 1998; Plattner et al., 1999; Woodring et al., 2002]. In nontransformed cells, the activation status of c-Abl is tightly controlled and its PTK activity is retained in an inactive conformation through intramolecular c-Abl interactions [Woodring et al., 2002; Hunter, 2007]. Indeed,

Fig. 1. c-Abl antagonism fails to inhibit mammary tumor growth in mice. Female Balb/c mice were injected orthotopically with syngeneic 4T1 cells and treated daily beginning on day 8 after engraftment with either vehicle or imatinib (50 mg/kg/day) as indicated. Primary tumors were removed surgically and weighed at days 24 and 27 after engraftment. Bars indicate the mean tumor weight per group (6 mice/group). Reprinted with kind permission from Allington et al. [2009].

displacing either the N-terminal autoinhibitory Cap region or the SH2/SH3 domains of c-Abl by the binding of its substrates or effector molecules rapidly and transiently stimulates this PTK, whose activity is downregulated following its ubiquitination and proteosomal degradation [Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004]. Cytoplasmic c-Abl is activated by a variety of growth factors and cytokines, by reactive oxygen species, and by cell attachment and mechanotransduction that directs c-Abl to adherens complexes and regions of actin cytoskeletal remodeling [Woodring et al., 2002; Zhu and Wang, 2004]. Nuclear c-Abl is also activated by reactive oxygen species and by DNA damage that couples c-Abl to the regulation of cell survival and apoptosis [Agami et al., 1999; Vigneri and Wang, 2001; Truong et al., 2003; Chau et al., 2004]. Interestingly, both of the oncogenic forms of c-Abl, namely BCR-Abl and v-Abl, are unable to enter the nucleus, and their enforced nuclear expression induces apoptosis, not cellular transformation [Woodring et al., 2002; Zhu and Wang, 2004; Suzuki and Shishido, 2007]. Thus, the pathophysiological output of c-Abl activation ultimately reflects a conglomeration of the initiating signal, the cellular context and the cellular locale wherein c-Abl is stimulated.

Fig. 2. Schematic depiction of the functional domains of human (1a and 1b) and murine (I and IV) Abl isoforms. The N terminus of c-Abl contains either an autoinhibitory Cap region or a consensus motif for myristoylation (black), which is followed by SH3 (blue; colors refer to the online version only) and SH2 (pink) domains, which is followed by the catalytic PTK domain (kinase, yellow). The central region of c-Abl possesses 4 PXXPKX/R sequences (purple), 3 nuclear localization sequences (NLS, white), and 3 high-mobility group-like boxes (HLB, green). Finally, the C

terminus of c-Abl houses domains for binding globular (G, orange) and filamentous (F, gray) actin, as well as a nuclear export sequence (NES, brown). The oncogenic forms of Abl (v-Abl and BCR-Abl) contain modified N-terminal regions that disrupt the autoinhibitory functions normally mediated by the Cap region, which elicits constitutive PTK activity. The aberrant N terminus in v-Abl comprises a viral Gag sequence (light blue), while that in BCR-Abl comprises a portion of the N terminus of the BCR (red).

As mentioned above, the oncogenic potential of c-Abl was elucidated by the discovery that c-Abl can be fused to the BCR region on chromosome 22, an untoward translocation event that gives rise to BCR-Abl and its ability to induce CML development and progression [Druker, 2006; Hunter, 2007]. The synthesis and implementation of imatinib (Gleevec) to antagonize the phosphotransferase activity of Abl revolutionized the treatment of CML by eliciting response rates of about 98% in patients with the chronic phase of CML at the time of diagnosis [Mauro and Druker, 2001; Mauro et al., 2002; Druker, 2006]. In stark contrast to its causal role in initiating hematopoietic cancers, a definitive function for c-Abl in promoting the formation and progression of solid tumors, including those of the breast, remains an active and controversial area of research. For instance, early cell biology studies found that the phosphorylation of Crk by c-Abl inhibits fibroblast and carcinoma cell motility by preventing the formation of Crk:p130Cas complexes [Kain and Klemke, 2001; Kain et al., 2003]. In addition, hepatocyte growth factor signaling and its stimulation of thyroid cancer cell migration was potentiated signifi-

cantly in imatinib-treated cells as compared with their vehicle-treated counterparts [Frasca et al., 2001], suggesting that c-Abl activity suppresses carcinoma motility. With respect to cancers of the breast, c-Abl activation has been associated with enhanced breast cancer cell proliferation, invasion, survival and anchorage-independent growth [Plattner et al., 1999; Srinivasan and Plattner, 2006; Lin and Arlinghaus, 2008; Srinivasan et al., 2008], and with their transformation by Src [Sirvent et al., 2007]. In stark contrast, c-Abl was observed to be essential in suppressing mammary tumorigenesis mediated by ephrin B2/ephrin B [Noren et al., 2006]. Moreover, we recently discovered that constitutive c-Abl activity abrogates the oncogenic behaviors of TGF- β in late-stage breast cancer cells, resulting in their phenotypic and morphologic reversion both in vitro and in vivo (see below) [Allington et al., 2009]. These latter findings suggest that imatinib administration may be contraindicated in breast cancer patients. Accordingly, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression have met with disappointing results, including the presence of severe drug

Table 1. Role of c-Abl in solid tumor progression

Study type	Disease/cells	Major finding	Reference
<i>Abl as a tumor promoter</i>			
Preclinical	lung cancer	increased c-Crk expression is associated with aggressive phenotypes	Miller et al., 2003
Preclinical	lung cancer	imatinib and Fus1 inhibit c-Abl and anchorage-independent growth of H1299 cells	Lin et al., 2007b
Preclinical	breast cancer	constitutive c-Abl activation is elicited by dysregulated EGFR, HER2 and Src, leading to increased cell invasion	Srinivasan and Plattner 2006
Preclinical	breast cancer	c-Abl mediates Src-transformation and survival of breast cancer cells	Sirvent et al., 2007
Preclinical	breast cancer	c-Abl mediates IGF-1 receptor-stimulated breast cancer progression	Srinivasan et al., 2008
<i>Abl as a tumor suppressor</i>			
Preclinical	colon cancer	c-Abl activates p73 α /GADD45 α , leading to apoptosis in response to DNA mismatch repair	Li et al., 2008
Preclinical	colon cancer	c-Abl activates p73 α /GADD45 α , leading to G2 arrest after induction of DNA mismatch repair	Wagner et al., 2008
Preclinical	breast cancer	ephrin B2/ephrin B4 suppress breast cancer tumorigenicity via activation of a c-Abl/Crk/MMP-2-signaling axis	Noren et al., 2006
Preclinical	thyroid cancer	imatinib enhances thyroid cancer cell motility in response to HGF	Frasca et al., 2001
Preclinical	breast cancer	activated c-Abl suppresses oncogenic TGF- β signaling, inhibits EMT and reverts breast cancer tumorigenicity in vitro and in vivo	Allington et al., 2009
Clinical phase I	breast cancer	imatinib offered no clinical benefit in PDGF receptor-positive metastatic breast cancer	Cristofanilli et al., 2008
Clinical phase II	breast cancer	imatinib provided no therapeutic benefit against invasive breast cancer patients	Modi et al., 2005
Clinical phase II	breast cancer	imatinib and capecitabine treatment failed to improve the clinical course of metastatic breast cancer patients	Chew et al., 2008
Clinical phase I/II	prostate cancer	imatinib administration either alone or in combination promoted disease progression and severe toxicity	Lin et al., 2006, 2007a
Clinical phase II	pancreatic cancer	imatinib administration fails to offer any therapeutic protection against pancreatic cancer	Chen et al., 2006; Gharibo et al., 2008

EGFR = Epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2; IGF-1 = insulin growth factor 1; MMP-2 = matrix metalloproteinase 2; HGF = hepatocyte growth factor; PDGF = platelet-derived growth factor.

toxicities and the initiation of disease progression [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Similar detrimental clinical outcomes were observed in pancreatic [Chen et al., 2006; Gharibo et al., 2008] and prostate [Lin et al., 2006, 2007a] cancer patients subjected to imatinib administration.

Table 1 summarizes the dichotomous roles of c-Abl in regulating solid tumor development and progression, and in doing so, highlights the need to identify novel biomarkers capable of staging and stratifying cancer patients on the basis of their c-Abl expression and signaling profiles.

TGF- β Signaling and EMT

TGF- β is a pluripotent cytokine that plays essential roles in regulating mammalian development and differentiation, and in maintaining tissue homeostasis [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and

Akhurst, 2009; Tian and Schiemann, 2009b]. The versatile nature of TGF- β is emphasized by the fact that virtually all cells in the metazoan body are capable of both producing and responding to this cytokine. TGF- β is now recognized as a potent tumor suppressor that prevents the dysregulated growth and survival of cells, particularly those of epithelial, endothelial and hematopoietic origins [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. The process of tumorigenesis and its associated genetic, epigenetic and microenvironmental alterations enable early-stage cancer cells to inactivate the cytostatic activities of TGF- β through mechanisms that remain incompletely understood. As these neoplastic cells continue to evolve towards advanced malignancy, they ultimately acquire the ability to convert the cytostatic functions of TGF- β into those capable of driving neoplastic progression, including the induction of tumor growth, invasion and metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and Akhurst, 2009; Tian and Schiemann, 2009b]. The functional conversion

of TGF- β behavior during tumorigenesis is known as the 'TGF- β paradox', whose eventual interpretation and translation holds the key to developing novel chemotherapies capable of preferentially targeting the oncogenic activities of TGF- β [Schiemann, 2007].

An important consequence of TGF- β signaling is its potential to induce EMT, a process whereby immotile, polarized epithelial cells transdifferentiate into highly motile, apolar fibroblastoid-like cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Essential features exhibited by epithelial cells undergoing EMT include (1) diminished cell polarity owing to the downregulated expression of epithelial cell markers (e.g., E-cadherin, zona occludens 1 and β_4 -integrin); (2) remodeled actin cytoskeletal architectures; (3) upregulated expression of fibroblast markers and genes operant in cell motility and invasion (e.g., vimentin, N-cadherin, α -smooth muscle actin, Twist); and (4) acquired expansion of cells that possess stem cell-like properties and phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Importantly, recent studies by our group have shown that the initiation of oncogenic TGF- β signaling coincides with its stimulation of EMT in normal and malignant MECs [Galliher and Schiemann, 2006, 2007; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b], suggesting that the eventual development and implementation of pharmacological agents capable of uncoupling TGF- β from EMT may one day improve the clinical course of breast cancer patients.

The ability of TGF- β to induce EMT commences upon its binding to the TGF- β type II receptor, which then recruits, transphosphorylates and activates TGF- β type I receptor, which then phosphorylates and stimulates the latent TGF- β transcription factors, Smad2 and Smad3. Following their activation, Smad2/3 interact physically with the co-Smad, Smad4, which enables the resulting heterocomplexes to translocate to the nucleus where they associate with a variety of transcriptional activators and repressors to govern the expression of TGF- β -responsive genes in a cell- and context-specific manner [Heldin et al., 2009; Wendt et al., 2009a]. The coupling of TGF- β to Smad2/3 stimulation, which is commonly referred to as either 'Smad2/3-dependent' or 'canonical' TGF- β signaling, plays an essential role in governing all aspects of the pathophysiological activities of TGF- β , including its induction of EMT [Oft et al., 1996; Valcourt et al., 2005; see also Masszi and Kapus, 2011, this issue]. In addition to its stimulation of canonical Smad2/3 signaling, we and oth-

ers have identified a variety of noncanonical effectors whose activation by TGF- β also mediate fundamental functions of this cytokine [for review, see Lamouille and Derynck, 2011, this issue]. With respect to its induction of EMT, TGF- β must also activate (1) MAP kinase family members, particularly ERK1/2 [Xie et al., 2004] and p38 MAPK [Bhowmick et al., 2001; Galliher and Schiemann, 2007; Galliher-Beckley and Schiemann, 2008]; (2) focal adhesion complex proteins, including β_1 - and β_3 -integrins [Bhowmick et al., 2001; Galliher and Schiemann, 2006], Src [Galliher and Schiemann, 2006, 2007], focal adhesion kinase [Wendt and Schiemann, 2009] and p130Cas [Wendt et al., 2009b]; (3) nuclear factor- κ B [Huber et al., 2004; Neil and Schiemann, 2008] and its downstream effector, cyclooxygenase 2 [Neil et al., 2008], which promotes EMT by initiating an autocrine prostaglandin E_2 :EP $_2$ receptor signaling loop [Tian and Schiemann, 2009a]; (4) phosphoinositide 3-kinase and its downstream effectors, AKT and mTOR [Bakin et al., 2000; Lamouille and Derynck, 2007; Lamouille and Derynck, 2011]; (5) small guanosine triphosphate-binding proteins, including cdc42, Rac1 and RhoA [Wendt et al., 2009a]; and (6) PAR6 and its recruitment of the E3 ligase, Smurf1 [Ozdamar et al., 2005]. Although the precise contribution of canonical and noncanonical TGF- β signaling in mediating the various subtypes of EMT has yet to be clarified, it is known that the activation of both pathways is necessary for the faithful initiation and completion of EMT by TGF- β and its ability to confer stem cell-like properties to epithelial cells, whose newfound plasticity enables metastatic cancer cells to thrive in otherwise hostile secondary sites [Polyak and Weinberg, 2009]. Readers desiring more in-depth discussions of the molecular mechanisms that underlie the ability of TGF- β to induce EMT are directed to several recent and comprehensive reviews [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009; Lamouille and Derynck, 2011].

c-Abl Activation Suppresses EMT and Breast Cancer Progression

An essential component of EMT centers on the dissolution of adherens junctions and the remodeling of the actin cytoskeleton, both of which enable transitioning cells to acquire motile and invasive phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Interestingly, c-Abl activity has been associated with both the assembly and dissolution of adherens junctions and with altered actin dynamics and architectures through its

ability to bind globular and filamentous actin [Woodring et al., 2001, 2002, 2004; Zandy et al., 2007; Zandy and Pendergast, 2008]. Thus, the process of EMT presents a unique situation in which the paradoxical functions of c-Abl and TGF- β may intersect during mammary tumorigenesis. For instance, c-Abl essentially governs all biological decisions made by cells, including whether they proliferate, migrate or invade, or even whether they live or die [Zhu and Wang, 2004; Lin and Arlinghaus, 2008]. In fact, the physiological functions performed by c-Abl in many ways parallel those played by TGF- β , including their capacity to serve as tumor suppressors or promoters in a cell- and context-specific manner [Masague, 2008; Tian and Schiemann, 2009b]. Given the obvious pathophysiological similarities that exist between TGF- β and c-Abl in epithelial cells, we hypothesized c-Abl as an essential player in determining the morphologies and phenotypes of MECs, including their ability to undergo EMT in response to TGF- β . We tested this hypothesis by manipulating the expression or activity of c-Abl via several complementary approaches: (1) loss of c-Abl function by pharmacological inhibition (i.e. imatinib), by retroviral transduction of a kinase-dead c-Abl mutant or by lentiviral transduction of a shRNA against c-Abl; or (2) gain of c-Abl function by retroviral transduction of a constitutively active c-Abl mutant (CST-Abl). These c-Abl manipulations were applied to 2 murine MEC cell lines to interrogate the potential linkage between c-Abl and TGF- β : (1) normal murine NMuMG mammary gland cells, which are nontransformed and exhibit normal cuboidal epithelial architectures that readily undergo a robust EMT in response to TGF- β [Sokol et al., 2005; Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Wendt and Schiemann, 2009; Wendt et al., 2009b]; and (2) malignant, metastatic 4T1 cells, which are a late-stage model of TGF- β -responsive breast cancer [Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil and Schiemann, 2008; Yang et al., 2008; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]. Figure 3a shows that c-Abl expression and activity were essential for maintaining normal MEC morphology, such that measures resulting in a loss of c-Abl function elicited an EMT response, while those measures resulting in a gain of c-Abl function produced a 'hyper-epithelial' response that was resistant to EMT and invasion stimulated by TGF- β [Allington et al., 2009]. The morphological alterations induced by inactivating c-Abl also transpired in normal human MECs [Allington et al.,

Table 2. c-Abl deficiency elicits an EMT transcriptional signature in NMuMG cells

NMuMG cells		Average relative mRNA, %	
		(-) TGF- β	(+) TGF- β
E-cadherin	scram	100.0	58.1 $\pm$ 8.7
	shAbl	104.6 $\pm$ 8.7	47.6 $\pm$ 7.8
N-cadherin	scram	100.0	856.0 $\pm$ 14.6
	shAbl	345.9 $\pm$ 21.1	1,050.9 $\pm$ 47.4
Vimentin	scram	100.0	475.6 $\pm$ 40.9
	shAbl	1,135.1 $\pm$ 26.9	858.0 $\pm$ 28.9
Twist	scram	100.0	295.3 $\pm$ 33.2
	shAbl	569.5 $\pm$ 20.3	683.9 $\pm$ 49.8

Quiescent parental (scrambled shRNA, scram) or c-Abl-deficient (shAbl) normal murine mammary gland cells (NMuMG) were incubated in the absence or presence of TGF- β_1 (5 ng/ml) for 48 h, at which point total RNA was isolated and subjected to semi-quantitative real-time PCR analysis. Data are the mean ($\pm$ SE; n = 3) percent change in EMT marker gene expression relative to that observed in untreated parental normal murine mammary gland cells.

2009], and more importantly, gene expression analyses confirmed the ability of c-Abl deficiency to increase mesenchymal gene expression (table 2). Thus, c-Abl inactivation results in morphological, transcriptional and migrational changes suggestive of those observed during EMT stimulated by TGF- β .

Extending these analyses to metastatic 4T1 cells demonstrated that CST-Abl expression was sufficient in reducing cell scattering and promoting stronger cell-cell junctions in traditional 2D culture systems (fig. 3b). The morphological differences induced by c-Abl activation were greatly exaggerated when these same cells were propagated in compliant 3D organotypic cultures. Indeed, in stark contrast to the large and irregularly shaped organoids formed by parental and loss of c-Abl function 4T1 cells, those expressing CST-Abl formed dramatically smaller and perfectly spherical organoids that appeared to undergo a partial hollowing (fig. 3c, d). In addition, CST-Abl expression reinstated the cytostatic activities of TGF- β in 4T1 cells in part by (1) acting as a broad-spectrum suppressor of matrix metalloproteinase expression [Allington et al., 2009], and (2) overriding the tumor-promoting signals engendered by rigid microenvironments [Allington et al., 2009]. Thus, enforced activation of c-Abl in metastatic MECs may provide a novel means to alleviate the oncogenic activities of TGF- β and, consequently,

(For legend see next page.)

to phenotypically and morphologically normalize the malignant behaviors of breast cancer cells.

We tested the validity of the above supposition by orthotopically engrafting parental and CST-Abl-expressing 4T1 cells in syngeneic Balb/c mice. As expected [Gallagher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Wendt and Schiemann, 2009; Wendt et al., 2009b], parental 4T1 cells rapidly formed palpable tumors that necessitated host euthanization by day 28 due to excessive tumor burden (fig. 4a). Remarkably, every animal injected with CST-Abl-expressing 4T1 cells failed to develop palpable tumors during the course of the study (fig. 4a) and to exhibit overt signs of disease during necropsy [Allington et al., 2009]. Surprisingly, clonogenic assays facilitated the reisolation CST-Abl-expressing 4T1 cells from the mammary fat pads of mice that were euthanized on day 51 (fig. 4b). Collectively, these findings suggest that measures capable of enforcing c-Abl activation may represent a novel means to abrogate the oncogenic activities of TGF- β in cancers of the breast, and as such, to one day to improve the prognosis and treatment of patients with metastatic breast cancer. c-Abl may also influence the latency and dormancy of disseminated breast cancer in the form of micrometastases.

Chemotherapeutic Targeting of c-Abl in Breast Cancer: Friend or Foe?

Since the inception of the National Cancer Act of 1971, science and medicine have waged an all-out battle aimed at conquering cancer. Although considerable

progress has been achieved in terms of our understanding of the molecular mechanisms that underlie neoplastic development and progression, cancer itself remains a significant health concern and burden in the United States. In fact, 1 in 4 deaths in the United States results from cancer, which is also the leading cause of death in individuals younger than 85 years of age [Jemal et al., 2009]. Despite these grim statistics, overall cancer incidence and mortality rates have begun to decline over the last decade, including those for breast cancer which annually contributes to more than 40,000 deaths and 192,000 new invasive cases of this deadly disease [Jemal et al., 2009]. Continuing along this positive trend will require the development of new diagnostic and chemotherapeutic regimens, as well as the elucidation of new knowledge of how cancer cells acquire the 6 essential phenotypes, or hallmarks, necessary to become malignant. Included in this phenotypic list is the ability of cancer cells to (1) disregard cytostatic signals; (2) grow autonomously; (3) stimulate angiogenesis; (4) ignore apoptotic signals; (5) become immortal; and (6) invade and metastasize [Hanahan and Weinberg, 2000]. The inability of developing neoplasms to acquire each of these phenotypes prevents their conversion to aggressive states, suggesting that these cancer hallmarks represent various rate-limiting steps during malignant development. Although EMT is not a recognized hallmark of tumorigenesis, type 3 EMT is essential for the acquisition of invasive and metastatic phenotypes by cancer cells and their development of chemoresistance. Thus, pharmacological targeting of individual cancer hallmarks and EMT, both singly and in combination, may offer new inroads to effectively treat the development and dissemination of human malignancies, particularly those of the breast.

Our findings showing that the c-Abl activation circumvents and overrides the oncogenic activities of TGF- β in normal and malignant MECs are intriguing in terms of their scientific and biological significance. For instance, alterations within tumor microenvironments can either restrain or free breast cancer progression in a manner that mirrors the conversion of TGF- β function from a suppressor to a promoter of tumor invasion and metastasis [Bierie and Moses, 2006]. Moreover, mounting evidence indicates that TGF- β promotes breast cancer progression in part via its reprogramming of MEC microenvironments and their cellular architectures. In attempting to circumvent the oncogenic activities of TGF- β in mammary tumors, science and medicine have employed a 'TGF- β centric' approach that is likely to fail

Fig. 3. Constitutive c-Abl activity suppresses EMT and induces MET in metastatic MECs. KD-Abl = Kinase-dead c-Abl mutant; shAbl = c-Abl deficient. **a** Direct FITC-conjugated phalloidin immunofluorescence was performed to monitor the actin cytoskeletal architecture in c-Abl-manipulated normal murine mammary gland (NMuMG) cells, which readily acquired mesenchymal morphologies in loss of c-Abl function MECs. **b** Bright-field images of c-Abl-manipulated 4T1 cells grown in traditional 2D tissue culture systems. Gain of c-Abl function elicited an apparent MET in 4T1 cells. **c, d** c-Abl-manipulated 4T1 cells were propagated for 7 days in compliant 3D organotypic cultures prior to analyzing their growth and morphology by capturing bright-field images (**c**), or by staining with FITC-conjugated phalloidin and DAPI (**d**). Gain of c-Abl function suppressed acinar growth and promoted normal acinar development, including partial hollowing of the resulting organoids. All are representative of 2–3 independent experiments and were reprinted with kind permission from Allington et al. [2009].

Fig. 4. Enforced c-Abl activation abrogates the growth of metastatic MECs in mice. **a** Female Balb/c mice were injected orthotopically with syngeneic parental (i.e. empty vector) or CST-Abl-expressing 4T1 cells (10,000 cells/animal; 12 animals/group), whose ability to grow as tumors was measured using digital calipers over a period of 30 days. Reprinted with kind permission

from Allington et al. [2009]. **b** The mammary glands of mice injected with CST-Abl-expressing 4T1 cells were excised at day 51 after engraftment, and were subsequently dissociated enzymatically to produce a heterogeneous, single-cell suspension that was subjected to a clonogenic assay to reisolate reverted CST-Abl-expressing 4T1 cells.

clinically because targeted TGF- β therapies (i.e. both large and small molecule inhibitors) uniformly function as pan-TGF- β antagonists whose activities are subject to the phenomena underlying the ‘TGF- β paradox’ – i.e. the ability of mammary tumorigenesis to convert TGF- β from a tumor suppressor to a tumor promoter [Schiemann, 2007]. Pan-TGF- β antagonists are also inadequate in accounting for the pleiotropic functions of TGF- β in (1) governing MEC architectures and microenvironments, and (2) regulating tumor-associated stromal components. Thus, these findings underscore the necessity to design and implement rapid diagnostic tests capable of discriminating cancer patients most likely to benefit from targeted TGF- β therapies from those individuals most likely to be harmed by TGF- β antagonism.

A potential alternative to antagonizing all cellular responses to TGF- β may involve the implementation of a targeted approach that selectively inactivates specific noncanonical TGF- β effectors that preferentially promote its oncogenic activities. We have provided preclinical evidence that supports the therapeutic potential of this alternative approach (e.g., inactivation of $\alpha v \beta 3$ integrin, Src, focal adhesion kinase, nuclear factor- κB , cyclooxygenase 2, or EP₂ receptor) [Galliher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Tian and

Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]; however, complete disease resolution has yet to be achieved using these applications due to their inability to phenotypically normalize and revert malignant MEC behaviors, architectures and microenvironments. Our findings demonstrate that the enforced activation of c-Abl can fulfill this latter requirement, and in doing so, can promote the phenotypic normalization and reversion of highly malignant, late-stage breast cancers in mice (fig. 4) [Allington et al., 2009]. To our knowledge, c-Abl activation represents the first bona fide tool competent to ablate the oncogenic activities of TGF- β , thereby restoring its cytostatic function in normalized and reverted MECs. Indeed, a bold extension of our findings leads us to propose that the development and implementation of allosteric c-Abl activators may one day provide a paradigm shifting the strategy to treat metastatic breast cancers. Clearly, the notion of chemically stimulating c-Abl is disconcerting to many scientists and clinicians, particularly since c-Abl has been linked to the oncogenic activities of the receptors for epidermal growth factor, platelet-derived growth factor and insulin-like growth factor 1, to the transforming activities of Src and signal transducer and activator of transcription 3, and to the prosurvival activities of ERK5 [Plattner et al., 1999; Srinivasan and Plattner, 2006; Sir-

vent et al., 2007; Lin and Arlinghaus, 2008; Srinivasan et al., 2008]. However, in all cases, it remains difficult to gauge the extent to which off-target effects of imatinib and other c-Abl inhibitors contribute to their apparent effectiveness against breast cancer cells in vitro. Moreover, data obtained in human clinical [Modi et al., 2005; Chen et al., 2006; Lin et al., 2006, 2007a; Chew et al., 2008; Cristofanilli et al., 2008; Gharibo et al., 2008] and murine preclinical trials (fig. 1) [Allington et al., 2009] clearly demonstrate the inability of imatinib to provide any chemotherapeutic benefit towards cancers of the breast. Along these lines, overexpression of c-Abl or the enforced nuclear expression of oncogenic Abl mutants (e.g., BCR-Abl or v-Abl) all fail to elicit cellular transformation [Zhu and Wang, 2004; Suzuki and Shishido, 2007], which points to the possibility that targeted c-Abl activation may in fact be well tolerated by normal MECs. Indeed, we find that MECs are exquisitely sensitive to subtle changes in c-Abl activity [Allington et al., 2009], and as such, we anticipate that even submaximal activation of c-Abl will prove sufficient to induce MET and suppress mammary tumorigenesis stimulated by TGF- β , thereby improving the clinical course of patients with metastatic breast cancer.

Unanswered Questions and Future Directions

Although our ability to accept or reject the aforementioned hypothesis clearly awaits additional experimentation in a variety of genetically distinct human breast cancer subtypes and tissues (e.g., luminal A vs. luminal B vs. ErbB2 vs. basal-like vs. normal-like) [Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003], our findings are nonetheless provocative and potentially paradigm changing with respect to redefining the role of c-Abl in regulating mammary gland development and neoplasia, and to establishing c-Abl as a novel suppressor of oncogenic TGF- β signaling in breast and other epithelial-derived cancers in part by inducing their phenotypic and morphologic normalization [Allington et al., 2009]. Figure 5 depicts our current understanding of the role of c-Abl in regulating normal and malignant MEC behavior and in suppressing the oncogenic activities of TGF- β . Interestingly, we find that inactivating noncanonical TGF- β effectors is sufficient in abrogating the ability of TGF- β to promote breast cancer progression in a manner somewhat reminiscent of that mediated by enforced c-Abl activation. These parallels raise a number of interesting questions, including (1) does c-Abl suppress mammary

tumorigenesis by inhibiting EMT, or by stimulating MET; (2) does enforced c-Abl activation alter the selection or expansion of breast cancer stem cells; (3) does cytoplasmic or nuclear c-Abl activation mediate its antitumor activities; (4) does the initiation of oncogenic TGF- β signaling uncouple c-Abl from activation by TGF- β ; and (5) do the tumor-suppressing activities of c-Abl require signaling inputs by TGF- β ? These questions and their potential for directing the future development and implementation of c-Abl chemotherapies during mammary tumorigenesis are discussed below.

Although c-Abl activation clearly promotes the acquisition of epithelial phenotypes in normal and malignant MECs (fig. 3) [Allington et al., 2009], it remains to be determined whether this event reflects the ability of c-Abl to inhibit EMT, or conversely, to stimulate MET. This question also bears important clinical relevance because inhibiting EMT or stimulating MET are both likely to be most effective in preventing the exit of metastatic cells from the primary tumor; however, the induction of MET may be contraindicated should this process be found to play an essential role in promoting the outgrowth of micrometastatic lesions. Our findings show that c-Abl deficiency or inactivation both elicit EMT, while c-Abl activation induces a 'hyperepithelial' morphology that normalizes and reverts the phenotypes of metastatic breast cancer cells (fig. 3, 4) [Allington et al., 2009]. Along these lines, epithelial cells naturally tend to drift and acquire mesenchymal characteristics in response to extended 2D culture durations, suggesting perhaps that the process of becoming 'mesenchymal' may reflect a more energetically favorable and stable state than that needed to become 'epithelial'. Alternatively, the nearly infinite stiffness of 2D culture systems may serve as an aberrant signal that drives epithelial cells to acquire mesenchymal-like properties as a means to compensate and survive in extremely rigid microenvironments [Butcher et al., 2009; Erler and Weaver, 2009]. In fact, we observed microenvironmental tension to be sufficient in overriding the cytostatic activities of TGF- β , an event that was circumvented by CST-Abl expression [Allington et al., 2009]. Thus, our findings are consistent with the notion that enforced c-Abl activation stimulates MET, as opposed to simply inhibiting EMT. Future studies need to thoroughly address this issue, as well as to explore the potential linkage between c-Abl and known inducers of MET, including Pax-2, Cdx2 and frizzled-7 [Hugo et al., 2007].

Recently, human and mouse MECs were observed to acquire stem cell-like properties when stimulated to un-

Fig. 5. Schematic depiction of the role of c-Abl in suppressing EMT and oncogenic TGF- β signaling in normal and malignant MECs. In normal MECs, c-Abl activation (1) maintains adherens junction stability and cortical actin architecture; (2) mediates growth arrest in response to TGF- β and DNA damage; and (3) represses MMP expression and secretion. As developing mammary neoplasms become more and more malignant, oncogenic TGF- β signaling in conjunction with focal adhesion complex activation (e.g., β_3 -integrin/focal adhesion kinase/Src) may pro-

mote the degradation of c-Abl and its uncoupling from the tumor-suppressing activities of TGF- β . The loss of c-Abl function ushers in the initiation of EMT and its dissolution of adherens junctions, its production and activation of matrix metalloproteinases, and its circumvention of cytostasis induced by TGF- β and DNA damage, which collectively enhance breast cancer progression and metastatic dissemination to distant locales. RI/RII = TGF- β type I receptor/TGF- β type II receptor; E-cad = E-cadherin; MDM2 = murine double minute 2.

dergo EMT by TGF- β [Mani et al., 2008]. Mechanistically, upregulated Id1 expression may function in dictating whether TGF- β expands or contracts the pool of cancer stem cells [Tang et al., 2007], and consequently, whether TGF- β suppresses or promotes mammary tumorigenesis. Indeed, inhibiting TGF- β signaling pharmacologically in cancer stem cells elicited MET and their acquisition of less aggressive, more epithelial-like morphologies [Shipitsin et al., 2007]. Our demonstration that activated c-Abl phenotypically and morphologically reverts the malignant behaviors of late-stage breast cancer cells (fig. 3, 4) [Allington et al., 2009] raises 2 interesting questions – does enforced c-Abl activation suppress the selection and expansion of cancer stem cells, and conversely, does imatinib administration promote solid tumor progression by enlarging the population of stem cell-like progenitors in post-EMT carcinoma cells? Thus, fu-

ture studies clearly need to investigate these important issues as well.

As mentioned above, the strong epithelial morphologies and phenotypes induced by c-Abl activation suggest that this PTK stimulates MET, presumably by functioning in the cytoplasm to affect adherens junctions and cytoskeletal architectures. However, in response to diverse extracellular stimuli (integrins), c-Abl is translocated to the nucleus where it functions in regulating DNA damage and mismatch repair responses [Lewis et al., 1996; Baskaran et al., 1997; Taagepera et al., 1998]. Moreover, c-Abl activation during DNA damage-induced apoptosis requires its coupling to the p53 relative, p73 [Yuan et al., 1999; Shaul, 2000]. Thus, the relative contributions of cytoplasmic and nuclear c-Abl in suppressing mammary tumorigenesis remains an important and unanswered question. With this idea in mind,

we observed the expression of CST-Abl to greatly enhance the induction of p21 expression by TGF- β [Allington et al., 2009] and to induce 4T1 organoids to express robust quantities of p73, suggesting that the morphological reversion of 4T1 acinar structures is partially dependent upon nuclear c-Abl signaling inputs [Allington and Schiemann, unpubl. observation]. Accordingly, we have found that CST-Abl expression is sufficient in resensitizing 4T1 cells to death induced by the DNA-damaging agent, 6-thioguanine (Allington and Schiemann, data not shown). Thus, while 6-thioguanine has previously failed as a single-agent chemotherapeutic for breast cancer, our findings suggest that combining enforced c-Abl activation with 6-thioguanine or other DNA damage-inducing agents may offer new inroads to alleviating breast cancer progression.

Finally, we found that TGF- β administration leads to a very transient activation of c-Abl in MECs [Allington et al., 2009], which is rapidly followed by their degradation of c-Abl in a manner that coincides with initiation of EMT [Allington and Schiemann, unpubl. observation]. Based on these findings, we speculate that the end result of c-Abl activation by TGF- β results in the Src-dependent degradation of c-Abl [Allington and Schiemann, unpubl. observation; Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004], and consequently, in the acquisition of EMT phenotypes stimulated by TGF- β . This model clearly contrasts with the ability of TGF- β to couple to c-Abl activation via a phosphoinositide 3-kinase- and p21-activated kinase-2-dependent pathway in fibroblasts [Daniels et al., 2004; Wang et al., 2005; Wilkes and Leof, 2006]. Despite these disparate activities for c-Abl in MECs and fibroblasts, it remains plausible that the ability of TGF- β to induce EMT requires the inactivation and degradation of c-Abl in epithelial cells (fig. 5). Along these lines, we have recently observed TGF- β stimulation of EMT to drasti-

cally reduce the expression and activity of c-Abl in both the cytoplasm and nucleus of normal MECs. Moreover, the kinetics of c-Abl degradation mirrored that for the acquisition of focal adhesion complex signaling by TGF- β , and more importantly, chemotherapeutic targeting of focal adhesion complexes was sufficient in protecting c-Abl from degradation induced by TGF- β [Allington and Schiemann, unpubl. observation]. Interestingly, augmented [Gallier and Schiemann, 2006, 2007; Gallier-Beckley and Schiemann, 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b] and attenuated [Bhowmick et al., 2004a, 2004b; Cheng et al., 2005; Yang et al., 2008] TGF- β signaling in mammary carcinoma cells has been associated with disease progression, which raises a second interesting question – can c-Abl activation morphologically and phenotypically revert the malignant behaviors of breast cancer cells that can no longer respond to TGF- β ? Future studies need to address this important issue, as well as determine how EMT dictates the expression, activation and localization of c-Abl in normal and malignant MECs. Indeed, answering these important questions may provide novel information capable of one day (1) staging and stratifying the treatment of mammary carcinomas based on their c-Abl and TGF- β signatures; and (2) enhancing our understanding of the ‘TGF- β paradox’ in promoting metastatic disease in breast cancer patients.

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