C/EBPβ at the core of the TGFβ cytostatic response and its evasion in metastatic breast cancer cells

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Summary

Breast cancers may evade the growth-inhibitory action of TGFβ by accumulating defects of unknown nature that selectively eliminate cytostatic gene responses. We found the transcription factor C/EBPβ to be essential for TGFβ induction of the cell cycle inhibitor *p15INK4b* by a FoxO-Smad complex and repression of *c-MYC* by an E2F4/5-Smad complex in human epithelial cells. These cytostatic responses are selectively missing in metastatic breast cancer cells from half of the patients that we tested. The basis for this loss was traced to an excess of the C/EBPβ inhibitory isoform LIP. We suggest that C/EBPβ plays a key role in the coordination of TGFβ cytostatic gene responses, and its malfunction may trigger evasion of these responses in breast cancer.

Introduction

Transforming growth factor-β (TGFβ) is the most potent and widespread growth-inhibitory cytokine known in mammals (Bierie and Moses, 2006; Roberts and Wakefield, 2003; Siegel et al., 2003). Its cytostatic effect is based on elevating the expression of the CDK inhibitors p15INK4b, p21CIP1, or p57KIP2 and concurrently repressing MYC growth-promoting factors and ID antidifferentiation factors (Siegel and Massagué, 2003). Cancer cells may gain a selective advantage from losing these responses. Indeed, resistance to the growth-inhibitory action of TGFβ is considered a hallmark of cancer (Hanahan and Weinberg, 2000).

TGFβ is also a paradigm of functional versatility, controlling not only cell proliferation but also differentiation and stress responses, the production of secretory factors and matrix proteins, and the activity of many signaling pathways. This pleiotropic action has important implications in cancer. In a fraction of gastrointestinal and pancreatic cancers and a small proportion of other cancers, TGFβ resistance results from mutations that inactivate the TGFβ receptors or their Smad signal transducers (Derynck et al., 2001; Massagué et al., 2000). But TGFβ cytostatic gene responses may also be selectively eliminated by downstream defects of unknown nature that spare receptor and Smad functions. When this occurs, tumor cells may use their remaining TGFβ responses to their advantage during invasion (Derynck et al., 2001; Roberts and Wakefield, 2003), evasion of immune surveillance (Gorelik and Flavell, 2002; Thomas and Massagué, 2005; Wojtowicz-Praga, 2003), and metastatic colonization (Dumont and Arteaga, 2003; Kang et al., 2005; Roberts and Wakefield, 2003; Siegel and Massagué, 2003).

To trigger gene responses, the TGFβ receptor complex phosphorylates Smad2 and Smad3 transcription factors, which then translocate into the nucleus, bind Smad4, and associate with diverse DNA binding cofactors to target specific genes for regulation (Massagué et al., 2005). Repression of *c-MYC* and *ID1* is mediated by Smad complexes with E2F4/5 (Chen et al., 2002; Frederick et al., 2004) and ATF3 (Kang et al., 2003a), respectively, whereas Smad complexes with FoxO factors (FoxO1, FoxO3, and FoxO4) mediate the induction of *p21CIP1* (Seoane et al., 2004) and *p15INK4b* (Gomis et al., 2006). FoxO factors are critical players in cellular growth-inhibitory responses to stress, and determinants of organismal longevity (Brunet et al., 2004; Greer and Brunet, 2005; Lee et al., 2003; Libina et al., 2003).

Understanding how the selective elimination of TGFβ cytostatic responses is achieved in cancer has remained an elusive

SIGNIFICANCE

Tumor cells frequently evade the growth-inhibitory action of the cytokine TGFβ, thus gaining one of the hallmarks of cancer. We suggest a mechanistic explanation for this process in breast cancer. We found that two critical gene responses for the growth-inhibitory effect of TGFβ require the transcription factor C/EBPβ. An excess of the C/EBPβ inhibitor LIP appears to selectively limit these gene responses in metastatic cells from certain breast cancer patients. Thus, C/EBPβ may constitute a weak link in the TGFβ pathway, which breast cancer cells may exploit to skirt growth inhibition. Interventions that would restore C/EBPβ activity may resensitize these cells to the tumor-suppressive action of TGFβ.

goal. Loss of the ultimate effector of cytostatic inputs, the tumor suppressor Rb, confers resistance to TGFβ (Herrera et al., 1996; Laiho et al., 1990), but loss of other interconnected tumor suppressors such as p53, p16Ink4a, ARF, or p27Kip1 does not. Because of functional redundancies, TGFβ remains a potent growth inhibitor in cells that lack p15Ink4b (Latres et al., 2000) or the c-Myc response (Chen et al., 2002). However, breast cancer cell lines with a combined loss of these two gene responses evade the cytostatic action of TGFβ (Chen et al., 2001).

To identify links in the TGFβ cytostatic program that could be prone to disruption in cancer, we investigated the organization of Smad-dependent transcriptional events in epithelial cells, focusing particularly on cytostatic gene responses. An analysis of genes that are controlled by the FoxO-Smad combination has revealed that some of these genes, including *p15INK4b*, additionally require C/EBPβ for their response to TGFβ. Moreover, *c-MYC* repression also shares this requirement. C/EBPβ thus emerges as a key player in the TGFβ cytostatic program. C/EBPβ is a member of the basic leucine zipper family of transcriptional regulators and plays important roles in cell proliferation, differentiation, and oncogene-induced senescence through both positive and negative effects on gene expression (Begay et al., 2004; Grimm and Rosen, 2003; McKnight, 2001). Defects in C/EBPβ favor tumor progression in mouse models (Sebastian et al., 2005), and excess of the C/EBPβ inhibitory isoform LIP is implicated in breast cancer progression in mice (Zahnow et al., 2001) and humans (Milde-Langosch et al., 2003; Raught et al., 1996; Zahnow et al., 1997). Based on these clues, we went on to find that a high level of LIP is linked to a loss of TGFβ-dependent cytostatic responses in metastatic cells from breast cancer patients.

Results

Role of C/EBPβ in the induction of CDK inhibitors by TGFβ

The *p21CIP1* gene response is mediated by Smad3 and Sma4 in combination with FoxO factors (FoxO1, FoxO3, or FoxO4) (Seoane et al., 2004). *p15INK4b* was found to also be part of the subset of TGFβ target genes that are controlled by the Smad/FoxO combination (Gomis et al., 2006; and see below). In addition, *p15INK4b* and *p21CIP1* share the property of being inhibited by binding of a c-Myc-Miz1 complex to their proximal promoter region (Seoane et al., 2001, 2002; Staller et al., 2001) (Figure 1A). However, the Smad-responsive regions of the *p15INK4b* and *p21CIP1* promoters show some differences. The Smad binding region of the *p21CIP1* promoter comprises one forkhead binding element (FBHE) followed by three closely spaced Smad binding elements (SBEs) (Figure 1A) (Seoane et al., 2004). In the TGFβ-responsive region of the distal *p15INK4b* promoter (Seoane et al., 2001), we observed two separate SBEs, one flanked by a FBHE, forming the "Smad binding region 1" (SBR1), and the other flanked by a consensus binding site for C/EBPβ (SBR2; Figure 1A). All these elements are conserved between human and mouse (Figure 1A, bottom). Thus, the presence of a C/EBPβ binding element near an SBE distinguished the *p15INK4b* promoter from the *p21CIP1* promoter and was investigated further.

The human *C/EBPβ* locus encodes three isoforms, LAP1, LAP2, and LIP, by alternative usage of different start codons (refer to Figure 1E). LAP1 and LAP2 contain a dimerization and DNA binding domain and a transcription regulatory domain and function as dimeric transcriptional regulators. LIP lacks the regulatory domain and functions as a dominant-negative inhibitor of LAPs (Grimm and Rosen, 2003; Zahnow, 2002). To investigate the role of C/EBPβ in the *p15INK4b* response, we used a transcriptional reporter construct based on the −751/+70 region of the human *p15INK4b* promoter. This construct was responsive to TGFβ in wild-type but not FoxO-depleted HaCaT cells (Figure 1B). The activity of this construct was increased by overexpression of FoxO and LAP2, particularly when combined with Smad3 overexpression (Figure 1B). The LAP2 construct used in these experiments contains a conservative ATG to ATC mutation (Met to Ile) that eliminates the LIP translation start site, thus averting ectopic expression of LIP (Descombes and Schibler, 1991).

When placed in front of an adenovirus E1b minimal promoter, the isolated SBR1 (−538/−506) segment showed increased activity in the presence of overexpressed FoxO3 but not LAP2 (Figure 1C, left panel). A similar response was obtained with the Smad binding region of *p21CIP1* (Figure 1C, middle panel). In contrast, the *p15INK4b* SBR2 (−443/−385) segment showed increased activity in the presence of ectopic LAP2 but not FoxO3 (Figure 1C, right panel). Another family member, C/EBPα, increased the basal activity of the SBR2 construct but conferred little response to TGFβ, whereas C/EBPγ had no effect (Figure 1D). Mutations that disrupt the SBE or C/EBPβ sites in the SBR2 abrogated its response to TGFβ and LAP2 but not a TGFβ-independent response to C/EBPα (Figure 1D). Among different C/EBPβ isoforms, LAP2, but not LAP1 or LIP, mediated SBR2 activation (Figure 1E). Furthermore, overexpression of LIP abolished the effects of LAP2, LAP2 plus Smads, and TGFβ (Figure 1E).

C/EBPβ interaction with the *p15INK4b* promoter

Evidence of a TGFβ-directed interaction of the endogenous proteins with the *p15INK4b* promoter was obtained by chromatin immunoprecipitation (ChIP) analysis of relevant promoter regions (Figure 2A). TGFβ addition to cells rapidly induced the binding of Smad2/3, FoxO3, and C/EBPβ to the SBR1/2 region of the promoter (Figure 2B), as well as the binding of Smad4 and FoxOs 1 and 4 (data not shown). Note that the antibodies do not distinguish between Smad2 and Smad3. This binding was not observed with the proximal promoter region of *p15INK4b*, which served as an internal control (Figure 2B). This proximal promoter region binds c-Myc, which disappeared on TGFβ stimulation (Figure 2B; refer also to Figure 1A).

To verify that LAP2 can bind to the distal *p15INK4b* promoter region, we ectopically expressed Flag epitope-tagged proteins of interest in HaCaT cells. ChIP assays demonstrated a TGFβ-dependent binding of Flag-LAP2, Flag-Smad3, and Flag-FoxO3 to the distal region, but not the proximal region (Figure 2C). Coimmunoprecipitation assays demonstrated a TGFβ-dependent interaction between endogenous Smad4 and C/EBPβ as well as Smad2/3 (Figure 2D). An interaction between HA-tagged LAP2 and Flag-tagged Smads 2, 3, and 4, but not Flag-Smad1, was observed in transfected COS-1 cells (data not shown). Also, endogenous and direct interaction between Smads and C/EBP family members has been reported (Choy and Derynck, 2003). Taken together, these results suggest to us that Smad, FoxO, and C/EBPβ target the SBR1/SBR2 region

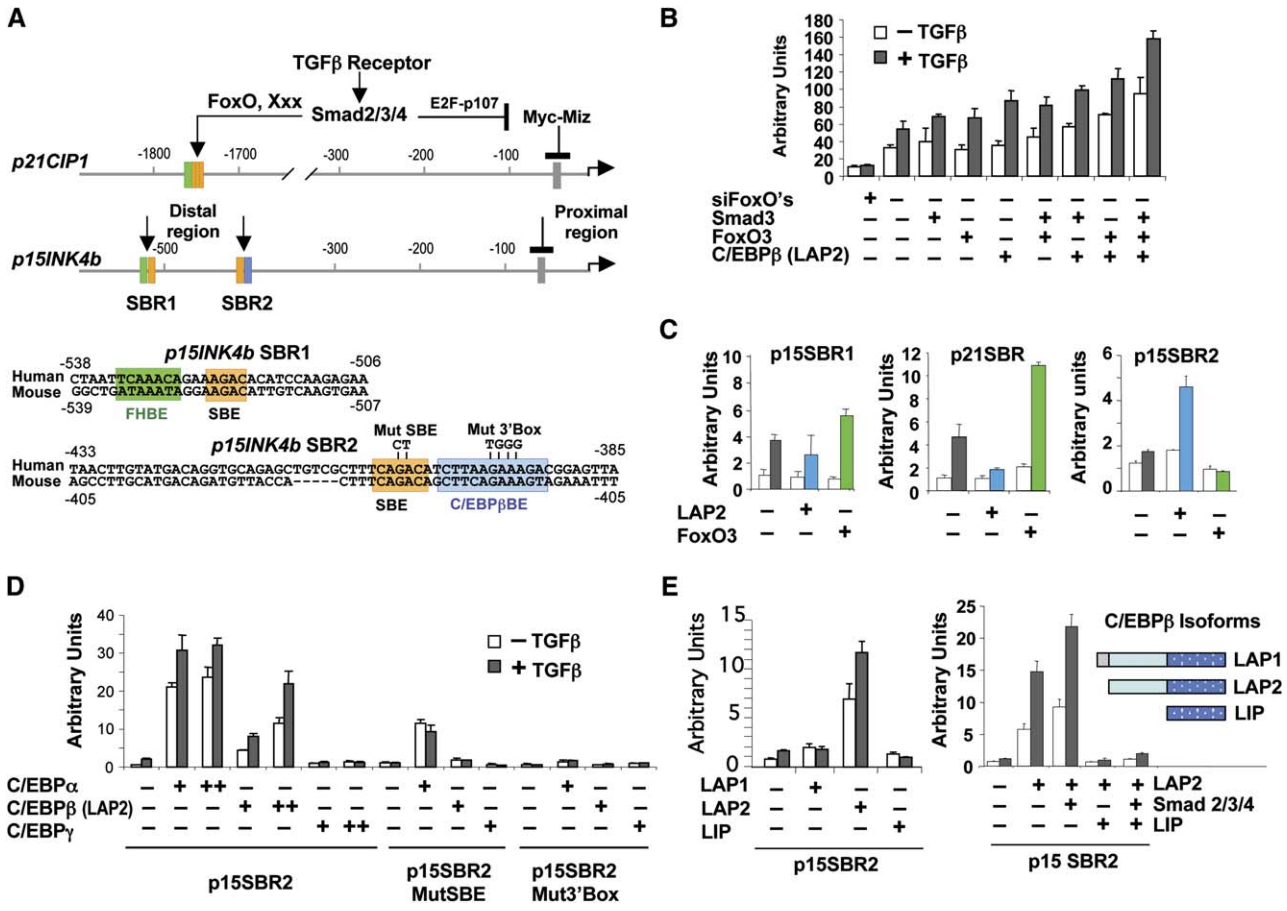

Figure 1. Smad, FoxO, and C/EBPβ transactivate p15INK4b

A: Graphic comparison of the *p15INK4b* promoter with the *p21CIP1* promoter and the dual TGFβ input. The nucleotide sequence of the human and mouse *p15INK4b* Smad binding regions (SBR1 and SBR2) containing binding elements for Smad (SBE, orange), Forkhead (FHBE, green), and C/EBPβ (C/EBPβBE, blue) are shown.

B: HaCaT cells were transfected with transcriptional reporter construct based on the −751/+70 *p15INK4b* promoter region driving firefly *luciferase* expression. Where indicated, the cells were cotransfected with siRNA against FoxOs, or with vectors encoding FoxO3, C/EBPβ LAP2, or Smad3. Cells were then treated with TGFβ for 20 hr, and luciferase activity was determined. Luciferase activity was normalized based on a cotransfected CMV-*renilla* luciferase vector. Data are mean ± SD (n = 3).

C–E: HaCaT cells transfected with *luciferase* reporter constructs driven by 4× multimers of p15SBR1, p15SBR2, and p21SBR (**C**), 4× multimers of p15SBR2 and the indicated mutations (**D**), or 4× multimers of p15SBR2 (**E**). FoxO, C/EBPβ, or Smad expression vectors were cotransfected with the reporter constructs as indicated. Cells were then treated as in **B**.

of the *p15INK4b* promoter to mediate TGFβ-dependent transcriptional activation.

C/EBPβ couples *p15INK4b* activation and *c-MYC* repression by TGFβ

To determine whether C/EBPβ is required for *p15INK4b* induction by TGFβ, we transfected HaCaT cells with a LIP expression vector. LIP overexpression markedly blunted the p15INK4b response at the mRNA and protein levels, with little effect on the p21CIP1 response (Figure 3A). In a separate approach, we also tested the effect of the stable knockdown of C/EBPβ by means of a shRNA vector (Figures 3B and 3C). C/EBPβ knockdown sharply inhibited the p15INK4b response with little effect on the p21CIP1 response (Figure 3D).

Together with the induction of CDK inhibitors, the transcriptional repression of *c-MYC* is an important part of the growth-inhibitory response of epithelial and hematopoietic cells to TGFβ (Scandura et al., 2004; Siegel and Massagué, 2003). The induction of *p15INK4b* and *p21CIP1* and the repression of *c-MYC* are concurrent events in HaCaT cells and are mediated by distinct Smad complexes. Given the opposite nature of the *c-MYC* and *p15INK4b* responses, we were surprised to find that both the overexpression of LIP (Figure 3A) and the knockdown of C/EBPβ (Figure 3D) inhibited *c-MYC* repression by TGFβ in HaCaT cells.

Repression of *c-MYC* is mediated by the binding of a transcriptional repression complex containing Smad, E2F4/5, and p107 to the "TGFβ inhibitory element" (TIE) in the proximal promoter region (Chen et al., 2002; Frederick et al., 2004). Enforced overexpression of LIP increased the basal activity of this *c-MYC* promoter region in transcriptional assays and blunted the transcriptional repression of this promoter caused by TGFβ (Figure 4A). ChIP analysis demonstrated binding of endogenous C/EBPβ to the TIE region under basal conditions (Figure 4B), which is in agreement with a recent report (Sebastian et al., 2005). TGFβ addition caused a small increase in this binding

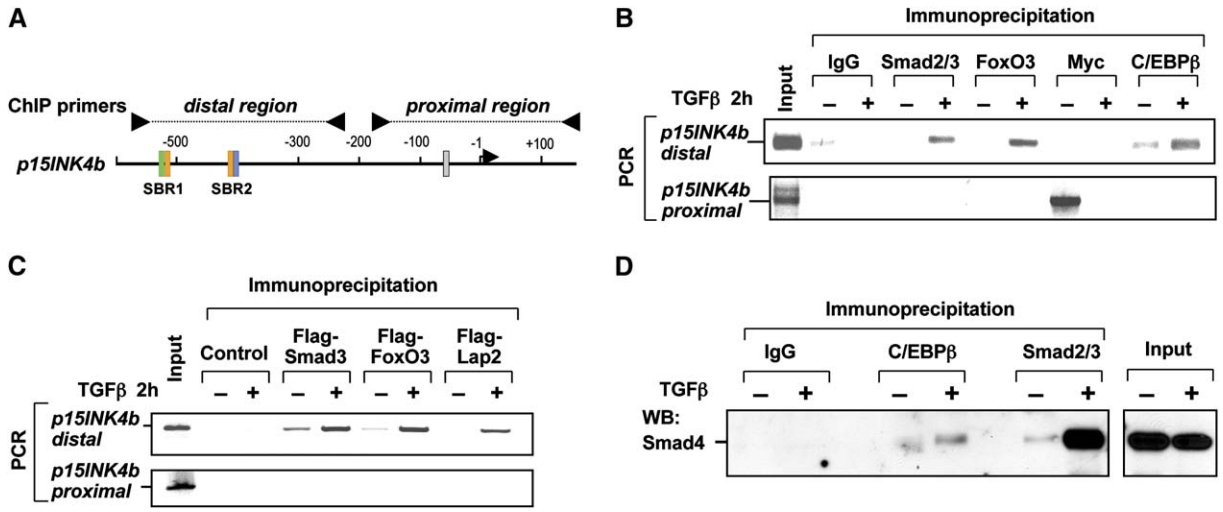

Figure 2. TGFβ-inducible binding of Smad, FoxO, and C/EBPβ to the *p15INK4b* promoter

A: A schematic representation of the human *p15INK4b* promoter regions amplified in ChIP assays. The SBEs (orange), FHBE (green), C/EBPβBE (blue), and INR (gray) are indicated.

B: HaCaT cells were incubated with or without TGFβ for 90 min and subjected to ChIP assays with the indicated antibodies and PCR primers. The proximal region of the *p15INK4b* promoter was used as a negative control.

C: HaCaT cells were transfected with the indicated Flag-tagged expression vectors and cultured for 24 hr. Cells were then incubated with or without TGFβ for 90 min and subjected to ChIP assays with anti-Flag antibodies and the indicated PCR primers.

D: HaCaT cells were treated with or without TGFβ for 90 min, and lysates were immunoprecipitated with the indicated antibodies and then subjected to immunoblotting with anti-Smad4 antibody.

(Figure 4B) together with a previously reported increase in the binding of Smad2/3 and E2F4 to this region (Chen et al., 2002).

Previous work has shown that depriving cells of either the p15Ink4b response (Latres et al., 2000) or the c-Myc response to TGFβ (Chen et al., 2002) does not diminish the growth-inhibitory effect of TGFβ. However, together with the blunting of both gene responses, the enforced overexpression of LIP in HaCat cells caused an attenuation of the growth-inhibitory effect of TGFβ (Figure 4C). The knockdown of C/EBPβ completely abolished the growth-inhibitory effect of TGFβ (Figure 4D). Collectively, these results show that C/EBPβ function is required for two gene responses—*p15INK4b* induction and *c-MYC* repression—that are central to the cytostatic action of TGFβ.

A LIP/LAP imbalance in TGFβ-resistant breast cancer cells

In light of these clues and previous work linking high LIP expression to breast cancer progression (Milde-Langosch et al., 2003; Raught et al., 1996; Zahnow et al., 1997), we wondered whether evasion of TGFβ cytostatic action in breast cancers may be

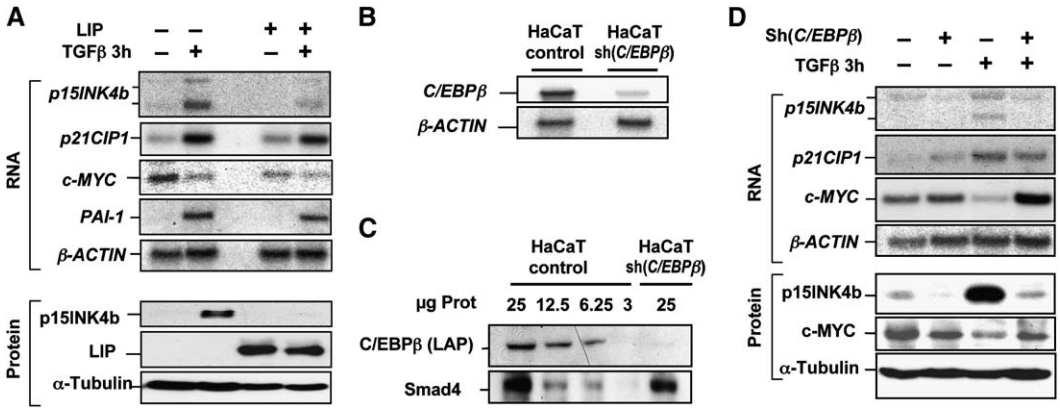

Figure 3. Requirement of C/EBPβ for cytostatic TGFβ gene responses

A: HaCaT cells infected with a retroviral vector encoding the LIP isoform of C/EBPβ or an empty retrovirus vector were incubated in the presence or absence of TGFβ for 3 hr. Total RNA was subjected to Northern blot analysis using the indicated probes. Whole-cell extracts from cells incubated with or without TGFβ for 5 hr were subjected to immunoblotting with the indicated antibodies.

B: Total RNA from control and C/EBPβ-depleted HaCaT cells was subjected to Northern blot analysis using the indicated probes.

C: Immunoblots with the indicated antibodies were performed on whole-cell extracts from control and C/EBPβ-depleted HaCaT cells.

D: Control and C/EBPβ-depleted HaCaT cells were treated with or without TGFβ for 3 hr. Total RNA was subjected to Northern blot analysis using the indicated probes. Whole-cell extracts were treated as in **A**.

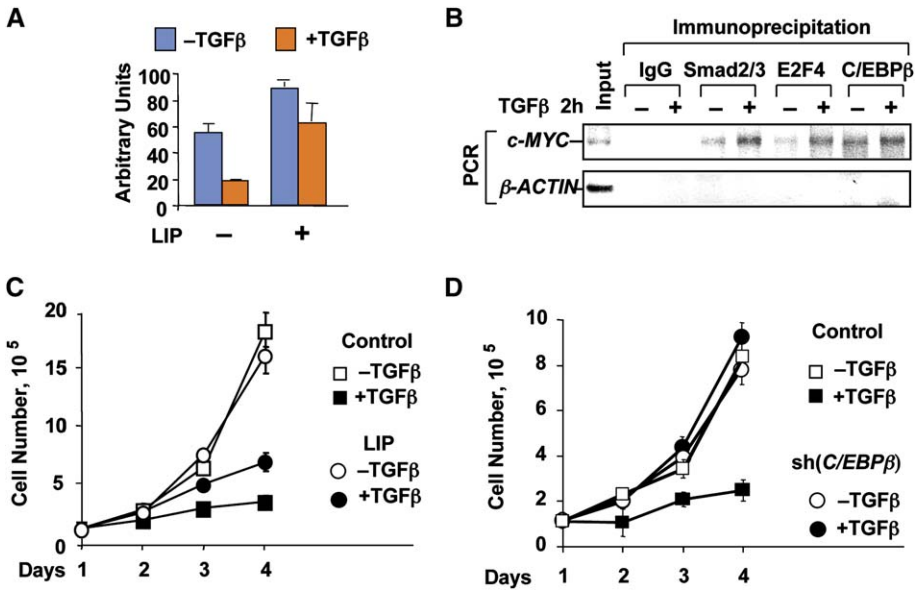

Figure 4. Requirement of C/EBPβ for the TGFβ growth-inhibitory response

A: HaCaT cells transfected with a firefly *luciferase* reporter construct driven by the TGFβ-responsive region of the *c-Myc* promoter plus a LIP expression vector as indicated were treated with TGFβ for 20 hr, and luciferase activity was determined. TGFβ-responsive luciferase activity was normalized against the activity of cotransfected renilla *luciferase* driven by a constitutive promoter. Data are mean ± SD (n = 3).

B: HaCaT cells were incubated with or without TGFβ for 90 min and subjected to ChIP assays with the indicated antibodies and PCR primers for the proximal region of the myc promoter. The *β-ACTIN* promoter was used as a negative control.

C and D: Growth curves of control and Lip-overexpressing HaCaT cells (**C**) or control and C/EBPβ-depleted HaCaT cells (**D**) with or without TGFβ. Data are average of triplicated cell counts ± SD.

linked to defects in C/EBPβ function. We investigated this possibility in malignant cells that were freshly obtained from the pleural fluid of patients with advanced breast cancer. These patients were undergoing therapeutic procedures as part of their routine clinical management at our institution. All samples were obtained and used under institutionally approved protocols. The cellular fraction from these fluids was enriched for carcinoma cells by positive selection using the epithelial cell marker EpCAM (Al-Hajj et al., 2003; Kielhorn et al., 2002; Latza et al., 1990). Of 42 samples that were collected and treated in this manner, eight yielded sufficient malignant cells for study (Table 1). These cells were propagated in culture for no more than 2 weeks prior to being used in experiments. Incubation with TGFβ led to the rapid accumulation of receptor-phosphorylated Smad2 and induction of the Smad target gene *CTGF* in all these samples (Figure 5A, middle panels), indicating that metastatic breast cancer cells frequently retain TGFβ receptor and Smad functions.

Four of the samples (CN19, CN30, CN43, and CN47) showed downregulation of *c-MYC* and induction of *p15INK4b* and *p21CIP1* in response to TGFβ (Figure 5A, top panels). These gene responses were accompanied by approximately 50% inhibition in the rate of DNA synthesis by TGFβ (Figure 5A, bottom panels). However, the other four samples showed defects in their cytostatic response to TGFβ. Depending on the specimen, these defects included an absence of *p15INK4b* response (sample CN34); absence of *p15INK4b* and *c-MYC* responses (samples CN37 and CN41); or absence of *p15INK4b*, *p21CIP1*, and *c-MYC* responses (sample CN46). The drop in DNA synthesis caused by TGFβ in these four samples barely surpassed 25%, and it was completely absent in sample CN46 (Figure 5A, bottom).

Western immunoblotting analysis of C/EBPβ products revealed striking differences in the level of LIP in these samples. LIP levels were at least 4-fold higher in the four samples that were defective in TGFβ cytostatic responses than in the TGFβ-sensitive samples (Figure 5B). When normalized to molar ratios, LIP was in molar excess over LAP in the samples lacking *c-MYC* and *p15INK4b* responses, and LAP was in molar excess in the samples with normal TGFβ responses. Thus, an inverse correlation exists between the LIP:LAP ratio in these breast cancer cells and their growth-inhibitory responsiveness to TGFβ (Figure 5C).

To investigate whether the defect in cytostatic TGFβ gene responses in breast cancer samples was accompanied by alterations in other TGFβ gene responses, we determined the level of expression of genes of interest in three LIP-rich samples (CN34, CN37, and CN41) and three samples with low LIP levels (CN19, CN43, and CN47). All six samples were derived from

Table 1. Clinicopathologic data of the primary tumors and metastases corresponding to the patients whose pleural fluid was used as a source of tumor cells in this study

Pleural sample	Type	Primary tumor size	Grade	ER	PR	HER2	Metastasis sites[a]
S19	ductal	3.5 cm	I/III	+	+	+	Pl, Lu, LN
S30	ductal	0.7 cm	III/III	–	–	+	Pl, LN, CW, Me, Ad
S34	ductal	2.5 cm	III/III	+	–	NA	Pl, Lu, LN, Bo
S37	ductal	3.2 cm	III/III	+	+	++	Pl, Lu, LN, Br, Li
S41	ductal	4.5 cm, 6.5 cm[b]	II/III	+	+	++	Pl, Lu?, Br, Bo, CW
S43	ductal	1.3 cm	II/III	+	+	+	Pl, Lu, Li, Bo
S46	ductal	0.7–3.8 cm	III/III	–	–	+++	Pl, Bo, ST, LN
S47	lobular	2 cm	NA	+	+	+	Pl, Lu, Li, LN, Ca, Co

[a]Pl, pleural; Lu, lung; LN, lymph nodes; CW, chest wall; Me, mesentery; Ad, adrenal; Bo, bone; Br, brain; Li, liver; ST, soft tissue; Ca, perinoteal carcinomatosis; Co, colon.
[b]Bilateral tumor.

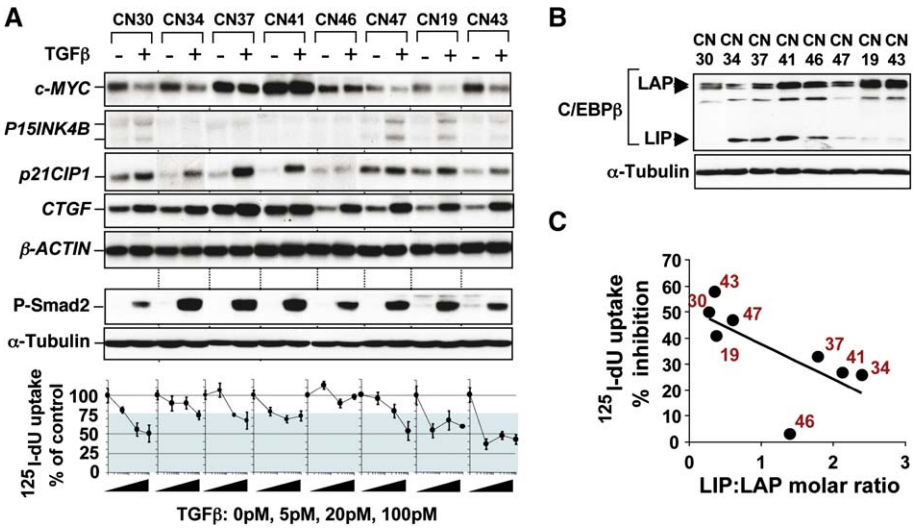

Figure 5. Evasion of TGFβ cytostatic responses in metastatic breast cancer cells is linked to LIP/LAP imbalance

A: EpCAM+ cells sorted from pleural effusion fluids from breast cancer patients were cultured in the presence or absence of TGFβ for 3 hr. Total RNA was subjected to Northern blot analysis using the indicated probes. Whole-cell extracts were probed with the indicated antibodies. Bottom: cells were treated for 20 hr with the indicated concentrations of TGFβ and pulsed for 6 hr with ^{125}I-deoxyuridine. ^{125}I-dU incorporation was determined, and the data are plotted as percentage incorporation relative to control cells that received no TGFβ. Data are mean ± SD (n = 3).

B: Whole-cell extracts from the cell samples described in **A** were probed with the indicated antibodies.

C: The relative inhibition of ^{125}I-dU incorporation by 100 pM TGFβ is plotted against the LIP:LAP molar ratio in each cell sample described in **B**.

patients with an initial diagnosis of ER+ ductal carcinoma (Table 1). In addition to lacking the *p15INK4b* response, the three LIP-rich samples lacked *GADD45A*, *LEMD3*, and *IER3* responses (Figure 6). Inductions of *GADD45A* and *LEMD3* by TGFβ have been identified as a FoxO- and C/EBPβ-dependent gene responses in human epithelial cells (Gomis et al., 2006). Induction of *SMAD7*, *PAI1*, *IL11*, *SGK*, *p21CIP1*, *GADD45B*, and *CTGF* by TGFβ was observed in most samples in both groups. However, *SMAD7*, *PAI-1*, and *GADD45B* were induced to lower levels in the LIP-rich group (Figure 6). In contrast, these three samples show a remarkably robust induction of *IL11*, which has been implicated in bone metastasis (Kang et al., 2003b, 2005). Collectively, these results show that a high level of LIP in metastatic breast cancer cells was associated with an attenuation of *p15INK4b* induction and other C/EBPβ-dependent responses, and an accentuation of *IL11* induction in response to TGFβ.

LAP restores TGFβ cytostatic responses in resistant breast cancer cells

To investigate the relationship between a high LIP:LAP ratio and a defective TGFβ cytostatic response, we sought to normalize this ratio in samples CN37 and CN41. We chose these samples because they are defective in both the *c-MYC* and *p15INK4b* responses to TGFβ but retain the *p21CIP1* response (refer to Figure 5B). As RNAi-mediated depletion of LIP is not feasible without also targeting LAP, we decreased the LIP:LAP ratio by means of a retroviral vector encoding human LAP2. Transduction of CN37 and CN41 cells with a LAP2 vector brought the LIP:LAP molar ratio from 1.8 and 1.6 to 0.4 and 0.3, respectively (Figure 7A). In CN41cells, LAP2 overexpression caused a reproducible decrease in the basal level of *c-MYC* mRNA and an increase in the basal level of *p21CIP1*, without evidence of Smad2 phosphorylation by autocrine TGFβ (Figure 7A).

LAP2 expression conferred to both samples the ability to downregulate *c-MYC* in response to TGFβ (Figure 7A). Quantitation of normalized *c-MYC* mRNA levels, from three independent experiments, demonstrated a TGFβ-induced decrease in *c-MYC* levels down to 46% and 68% of basal levels in CN37 and CN41 cells, respectively (Figure 7A). LAP2 additionally restored the *p15INK4b* response to TGFβ, as well as the response of other C/EBPβ-dependent members of the FoxO-Smad-responsive group, *GADD45A*, *LEMD3*, and *CDC42EP3* (Gomis

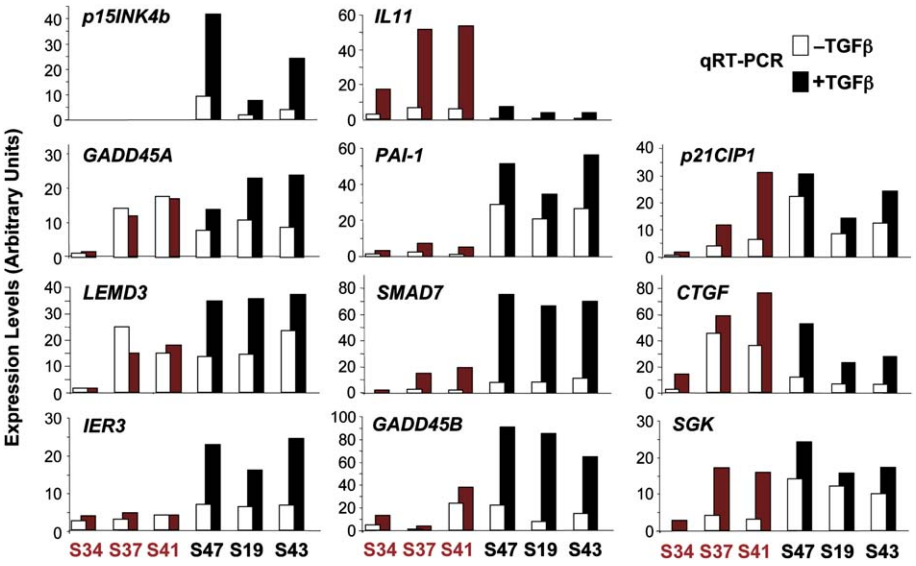

Figure 6. TGFβ responses in breast cancer cells

Expression levels of different TGFβ target genes in six primary breast cancer cell samples that were incubated with or without TGFβ for 3 hr and analyzed by qRT-PCR using primers for the indicated genes.

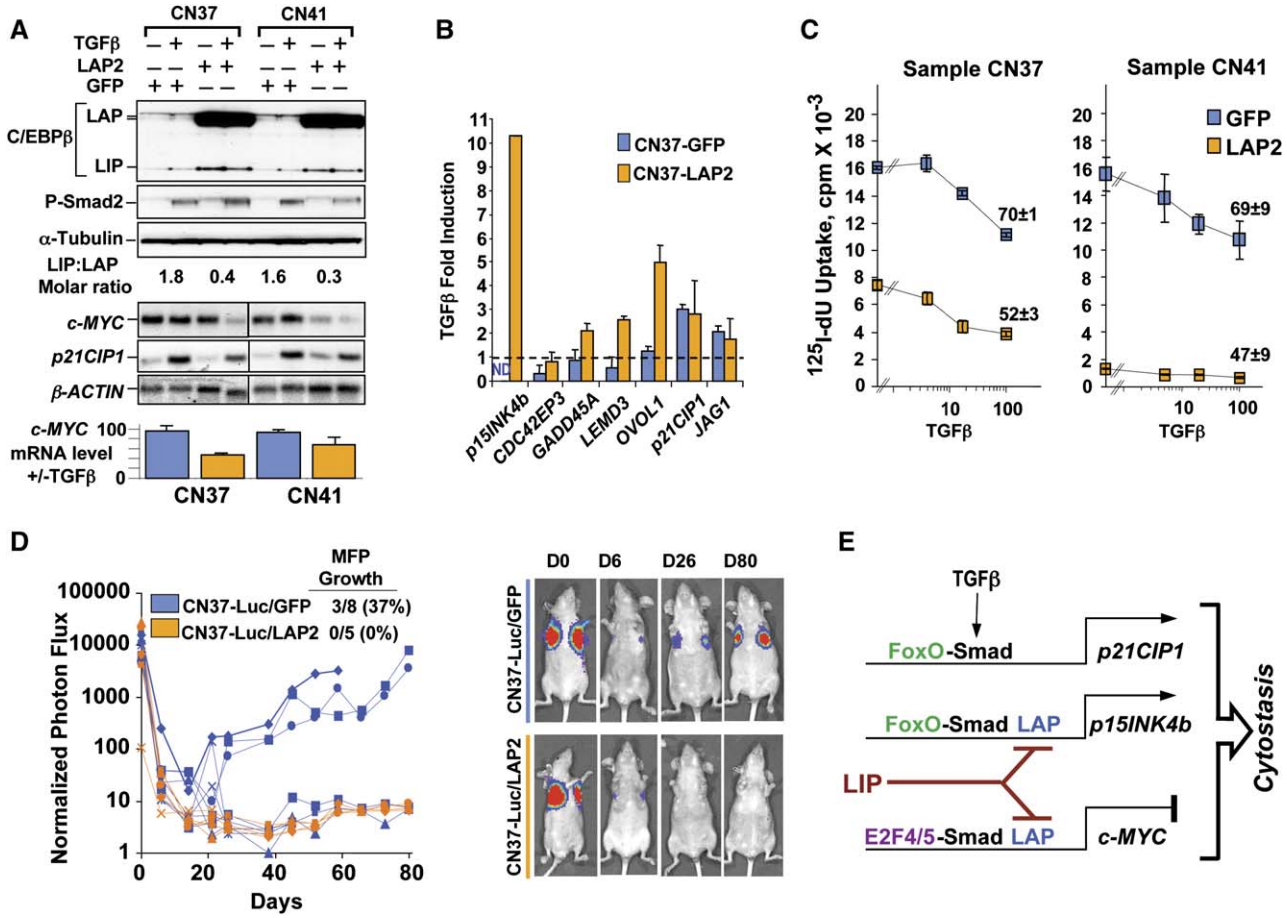

Figure 7. Lap restores TGFβ cytostatic responses in resistant breast cancer cells

A: Tumor cell samples CN37 and CN41 were infected with a retroviral vector encoding the LAP2 C/EBPβ isoform or a GFP fusion protein as control. Transduced cells were selected for 48 hr based on a puromycin resistance marker, allowed to recover for another 24 hr, and then treated with or without TGFβ for 3 hr. Total RNA was subjected to Northern blot analysis using the indicated probes. Whole-cell extracts were probed with the indicated antibodies. *c-MYC* levels were normalized against *β-ACTIN* and were used to calculate the TGFβ fold repression. Data are mean ± SD of three independent experiments.

B: Total RNA from sample CN37 in **A** was subjected to qRT-PCR analysis using the indicated probes. Data are mean ± SD of three independent experiments. *ND*, not detectable signal.

C: CN37 and CN41 cells treated as in **A** were then incubated for 20 hr with the indicated concentrations of TGFβ and cultured in the presence of ^{125}I-dU for an additional 6 hr. ^{125}I-dU incorporation into DNA was determined. Data are mean ± SD (n = 3). The ^{125}I-dU incorporation of the 100 pM TGFβ condition compared to controls that did not receive TGFβ is indicated for each cell sample.

D: Growth in mammary fat pad (MFP) of CN37 cells stably transfected with a retroviral construct encoding firefly luciferase and also stably transfected with control or LAP2 vector. Each curve shows tumor luminescence as a normalized photon flux emission. The mice images are representative of each group.

E: Schematic model of a set of cytostatic gene responses triggered by TGFβ in keratinocytes and primary breast cancer cells. Induction of *p21CIP1* and *p15INK4b* are mediated a by FoxO-Smad combination, whereas repression of *c-MYC* is mediated by a E2F4/5-Smad combination. The *p15INK4b* and *c-MYC* responses are enabled by the active C/EBPβ isoform LAP2, which is opposed by the natural dominant-negative inhibitory isoform LIP. In metastatic breast cancer cells with a high LIP:LAP ratio, the *p15INK4b* and *c-MYC* are cancelled. See the text for details and references.

et al., 2006), as determined by qRT-PCR in CN37-LAP2 (Figure 7B). LAP2 had little or no effect on the TGFβ response of *p21CIP1* (Figure 7A) and *JAGGED1* (Figure 7B), which is consistent with these being C/EBPβ-independent FoxO-Smad target genes. Thus, LAP2 overexpression rescued C/EBPβ-dependent TGFβ gene responses, including cytostatic responses, that were missing in these breast cancer cells.

LAP2 overexpression caused a significant reduction in the proliferative activity of CN37 and CN41 cells. The rate of DNA synthesis in LAP2-transduced CN37 and CN41 cells compared to controls dropped by approximately 50% and 90%, respectively (Figure 7C). The CN41-LAP2 cells, in particular, presented a flat, enlarged morphology typical of senescent cells, in contrast to the transformed morphology of control CN41 cells (data not shown). This morphology, together with the associated drop in proliferative activity and the changes in basal *c-MYC* and *p21CIP1* expression induced by LAP2, is suggestive of cell senescence. This notwithstanding, LAP2 overexpression increased the cytostatic response to TGFβ in CN37 cells, which responded with an approximately 50% inhibition in the rate of DNA synthesis (Figure 7C). Thus, restoration of a low LIP:LAP ratio rescued missing C/EBPβ-dependent TGFβ gene responses, including *c-MYC* and *p15INK4b*, together with a more extensive growth-inhibitory effect of TGFβ.

To determine whether LAP2 overexpression inhibited the tumor-initiating capacity of these cells in vivo, CN37 and CN37-LAP2 cells were transduced with a luciferase vector and implanted in the mammary fat pads of nude mice. The bulk of

the inoculated cell population died off during the first 10 days after implantation, as determined by quantitative bioluminescence imaging (Figure 7D). Over the next 80 days, CN37 cells formed tumors in three out of eight inoculated mammary fat pads, whereas the CN37-LAP2 cells failed to form tumors (Figure 7D). No remaining CN37-LAP2 cells could be detected in the mammary fat pads by tissue examination after sacrifice on day 80 (data not shown). Therefore, decreasing the LIP:LAP ratio not only restored C/EBPβ-dependent TGFβ gene responses and growth inhibition in the bulk tumor cell population but also inhibited the tumor-initiating capacity of CN37 cells.

Discussion

The present results have uncovered an unexpected role of the multifunctional transcription factor C/EBPβ in the coordination of key cytostatic gene responses to TGFβ. Furthermore, our results suggest a mechanism for the selective loss of these responses in metastatic breast cancers (summarized in Figure 7E).

C/EBPβ at the core of the TGFβ cytostatic program

The induction of CDK inhibitors by TGFβ and the repression of transcriptional enforcers of cell proliferation constitute, in different combinations, the cytostatic program triggered by this cytokine in epithelial and hematopoietic cells (Scandura et al., 2004; Siegel and Massagué, 2003). The induction of *p21CIP1* by TGFβ involves a FoxO binding site and three contiguous Smad sites (Seoane et al., 2004). In contrast, here we find that the induction of *p15INK4b* involves two separate Smad binding elements, one flanked by a FoxO site and the other by a C/EBPβ site. All these sites contribute to the TGFβ response. C/EBPβ binds to this region along with Smad and FoxO factors on TGFβ stimulation. C/EBPβ is essential for *p15INK4b* induction, as demonstrated by RNAi-mediated knockdown of C/EBPβ or overexpression of the naturally occurring C/EBPβ inhibitory isoform LIP.

We found that C/EBPβ is required not only for the induction of *p15INK4b* but also for the repression of *c-MYC*. Indeed, C/EBPβ is known to have opposite effects on gene expression, depending on the target (Begay et al., 2004; Grimm and Rosen, 2003; McKnight, 2001). Repression of *c-MYC* is not mediated by FoxO-Smad but by a E2F4/5-Smad combination (Chen et al., 2002; Frederick et al., 2004). In our ChIP assays, binding of C/EBPβ to the *p15INK4b* promoter is stimulated by TGFβ, whereas binding to the proximal *c-MYC* promoter seems to be partly constitutive. These results argue that C/EBPβ may use distinct mechanisms to enable different gene activation and repression responses in the Smad pathway. Ongoing work suggests that up to one-fifth of all TGFβ gene responses in keratinocytes require C/EBPβ (our unpublished data).

The requirement of C/EBPβ in both *p15INK4k* induction and *c-MYC* repression places this factor at the core of the TGFβ cytostatic program (Figure 7E). The growth-inhibitory effect of TGFβ in keratinocytes indeed is abolished by RNAi-mediated knockdown of C/EBPβ. C/EBPβ is implicated in growth inhibition and terminal differentiation in some contexts but stimulates cell proliferation in others (Begay et al., 2004; Grimm and Rosen, 2003; Lekstrom-Himes and Xanthopoulos, 1998). The basis for these opposite effects is unknown but could reflect differences in the LIP:LAP ratio (Zahnow et al., 2001). As shown here, the level of LIP might be an important determinant of the responsiveness of human keratinocytes and breast cancer cells to TGFβ. LIP translation is controlled by the RNA binding protein CUG-BP1 (Timchenko et al., 1999, 2001, 2005). Interestingly, epidermal growth factor signaling leads to CUG-BP1 phosphorylation and increases the LIP:LAP ratio (Baldwin et al., 2004). It will be important in the future to more completely define the role of C/EBPβ in TGFβ action and how these C/EBPβ regulatory inputs may become integrated with the Smad pathway.

C/EBPβ and evasion of TGFβ tumor suppression in breast cancer

Breast cancer cells that become resistant to the growth-inhibitory effects of TGFβ often retain TGFβ receptors and Smad functions but have a selective loss of cytostatic gene responses. This phenomenon was previously observed with genetically modified mouse models and human tumor-derived cell lines (Dumont and Arteaga, 2003; Kang et al., 2005; Roberts and Wakefield, 2003; Siegel and Massagué, 2003). This phenomenon has been validated in the present studies with primary metastatic breast cancer cells. Of eight clinical samples that we investigated, none shows defects in TGFβ receptor or Smad function. These cells responded to TGFβ with Smad phosphorylation and several Smad-dependent gene responses, such as induction of *CTGF*, *IL11*, *SMAD7*, or *p21CIP1*. However, half of these samples lacked *p15INK4b* and *c-MYC* responses and were only weakly growth inhibited by TGFβ. In samples that retain the *p15INK4b* and *c-MYC* responses, TGFβ inhibits proliferation by at least 50%. We do not know whether this extent of inhibition still represents a suboptimal response, but it certainly defines, within these metastatic samples, a group with higher sensitivity to cytostatic action of TGFβ.

The absence of *p15INK4b* and *c-MYC* responses in some of these primary breast cancer cells is striking for two reasons. First, this anomaly phenocopies the defects obtained by C/EBPβ depletion or LIP overexpression in keratinocytes, Secondly, this defect coincides with the presence of a high LIP:LAP ratio in the clinical samples that we examined. By transducing LIP-rich breast cancer cells with a LAP2 expression vector, we were able to recover the missing TGFβ gene responses and to decrease the tumorigenic activity of these cells. Thus, a link between a high LIP:LAP ratio and TGFβ resistance is apparent within these primary breast cancer cells. A high LIP:LAP ratio in human breast carcinomas correlates with a poorly differentiated phenotype of poor prognosis (Milde-Langosch et al., 2003; Raught et al., 1996; Zahnow et al., 1997). Mouse mammary tumors contain high levels of LIP (Zahnow et al., 2001), and overexpression of a LIP transgene in the mammary epithelium can cause hyperplasia (Zahnow et al., 2001). A loss of TGFβ tumor-suppressive function in LIP-rich tumors could partly account for these previous observations.

The central role of C/EBPβ in TGFβ-induced cytostasis and its malfunction in breast cancer may in turn be linked to phenomena of cell senescence under oncogenic stress. When we overexpressed LAP2 in two LIP-rich breast cancer cell samples, the cells underwent a decrease in proliferation that was partial in one sample and nearly complete with features of cell senescence in another. It has been reported that TGFβ signaling and Smad function are required for Ras-induced senescence in keratinocytes (Tremain et al., 2000; Vijayachandra et al., 2003), whereas C/EBPβ has been shown to mediate Ras-induced senescence by acting downstream or independently of the ARF-p53 pathway (Sebastian et al., 2005). These

phenomena could have a common basis in the role of C/EBPβ here uncovered.

In sum, our results establish a mechanistic link between C/EBPβ and TGFβ cytostatic gene responses in epithelial cells, and how disruption of this link may be at play in certain metastatic breast cancers. Studies with larger groups of patients are needed in order to establish the prevalence of TGFβ resistance in advanced breast cancer and the extent to which this resistance would be caused by a high LIP:LAP ratio. Our results also suggest a mechanism for how a high LIP:LAP ratio could contribute to an unfavorable evolution of breast cancers. By canceling cytostatic TGFβ gene responses, a LIP:LAP imbalance may set cancer cells free to co-opt for metastasis what remains of this pathway. For example, Smad-mediated induction of *CTGF* and *IL11* by TGFβ (Kang et al., 2003b, 2005) and increased production of parathyroid-related protein (Yin et al., 1999) are implicated in breast cancer bone metastasis. As shown here in preliminary form with patient-derived samples, the ability to reverse this imbalance may restore TGFβ tumor-suppressive functions in breast cancer.

Experimental procedures

Cell lines, transfection, and reporter assays

HaCaT keratinocytes cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). Cell culture media also contained 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 1 μg/ml fungizone. Cell lines were transfected with DNA using Lipofectamine following the manufacturer's instructions (Invitrogen). Luciferase reporter assays were performed as previously described (Seoane et al., 2001). A CMV-Renilla luciferase plasmid (Promega) was included to control for transfection efficiency. TGFβ1 (R&D Systems) was used at 100 pM.

Pleural effusion samples

Patients with metastatic breast cancer and pleural effusions who required thoracentesis for clinical indications were eligible to participate in this IRB-approved study. Informed consent was obtained prospectively at the time of the pleural fluid collection. A clinicopathologic database was created by extracting data from the patients' medical records (Table 1). Patient identifiers were removed from the samples to protect patient confidentiality as per institutional guidelines. Collections were done under sterile conditions. The samples received 5 U/ml of heparin and were centrifuged for 10 min at 1000 rpm at 4°C. The supernatant was discarded. If the pellet was bloody, 10 ml of ACK lysis buffer (Cambrex: 10-548) was added for 5 min at room temperature. Cells were suspended in 100 ml of PBS, centrifuged, and decanted. One-tenth of the cell pellet was frozen (DMSO 10%, FBS 90%) at −80°C. One-tenth was resuspended in 500 μl of PBS for cytospin analysis. The rest of the sample underwent negative selection using immunomagnetic beads to remove leukocytes. We used a magnetic particle concentrator (Dynal MPC-1) and anti-CD45 beads (Panleukocyte: Dynal Biotech) and CD15 beads (Myeloid cells: Dynal biotech), per the manufacturer's instructions. Cells were cultured for 24 hr and then incubated with a specific epithelial antibody (anti-EpCAM-APC) (Becton Dickinson) at a 1:50 dilution with PBS and 0.01% sodium azide. Unstained cells and isotype antibody (anti-IgG1-APC)-stained samples were used as controls. EpCAM-positive cells were sorted using a DakoCytomation MoFlo cell sorter. All pleural effusion cells were maintained at 5% CO_2 and 37°C in M199 media (Sigma) supplemented with 2.5% FBS, 10 μg/ml insulin (SIGMA), 0.5 μg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (Invitrogen), 100 ng/ml cholera toxin (Sigma), 1 μg/ml fungizone, and 100 U/ml penicillin/streptomycin. Cells were maintained in culture for a maximum of 12 days.

Plasmids

p15SBR1, p15SBR2MutSBE, and p15SBR2Mut3′box were constructed in pE1bTATALuc vector. Vectors encoding C/EBPβ and the three independent isoforms (LAP1, LAP2, and LIP) were generously provided by U. Schibler (University of Geneva). Human LAP2 and LIP were cloned by PCR from cDNA template and then digested with EcoR1/SalI and inserted in pBabe-Puro. Human LIP was cloned by PCR from cDNA templates and then subsequently digested with BamHI, inserted in pQCXIP (Clontech), and analyzed for the right orientation. pCDNA3-C/EBPα was generously provided by P.F. Johnson (NCI-Frederick). The C/EBPγ insert was subcloned into the EcoRI/SalI sites of a modified pCMV5 plasmid containing two copies of the HA epitope. A retroviral construct containing a triple fusion protein reporter construct encoding herpes simplex virus thymidine kinase 1, green fluorescent protein (GFP), and firefly luciferase was used as a transfection control (Minn et al., 2005). The Del-5 *c-MYC* reporter construct (Chen et al., 2001) and the p15SBR2 and Ha-FoxO constructs (Seoane et al., 2004) were previously described.

Small interfering RNA and short hairpin RNA

siRNA duplexes targeting FoxO1, 3, and 4 were obtained from the MSKCC High Throughput Screening Core Facility. The coding strands of the siRNA were as follows: FoxO1, CCGCGCAAGAGCAGCTCGT, TGTGCGCCTGGA CTCTTGA; FoxO3, GGGCGACAGCAACAGCTCT; and FoxO4, CCCGACCA GAGATCGCTAATT. Cell lines were transfected with siRNA using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen).

The siRNA-expressing H1 pRetrosuper retroviral system was generously provided by R. Bernards (Netherlands Cancer Institute) (Brummelkamp et al., 2002). The pRetrosuper sh(*C/EBPβ*) vector was generated by digesting pRetrosuper with BglII and HindIII and ligating with annealed oligos. The siRNA oligo pairs targeting *C/EBPβ* were 5′GATCCCCATCCATGGAAGTGG CCAAC TTCAAGAGAGTTGGCCACTTCCATGGATTTTTTGGAA. The target sequences are underlined. Ecotropic retroviral supernatants were harvested 48 hr posttransfection of Phoenix packaging cells, filtered (0.45 μM), and then concentrated at 18,000 rpm for 2 hr and used for overnight infections of HaCaT cells in the presence of 4 μg/ml of polybrene. After 24 hr of culture in fresh media, infected cells were selected with 5 μg/ml puromycin for 24–48 hr.

Northern blot

Cells were incubated with TGFβ as indicated, and total RNA was extracted using the Qiagen RNeasy kit. Total RNA (5 μg) was used for Northern blot analysis. RNA was fractionated through a 1% agarose gel and transferred to Hybond N+ nylon membranes (Amersham Pharmacia). Data were quantified with a STORM 840 scanner and Scanner Control v4.1 software (Molecular Dynamics).

Immunoprecipitation and Western immunoblotting

Immunoprecipitation assays were performed in binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 2 mM DTT, 10 mM NaF, 20 mM β-glycerophosphate, 0.05% NP-40, and protease inhibitor cocktail), using the same amount of cell extracts (1 mg protein) in all conditions. Antibodies used were anti-Smad2/3 (Kretzschmar et al., 1999), anti-C/EBPβ (C-19, Santa Cruz), and rabbit IgG (Cell Signaling). Anti-rabbit Ig beads and rabbit IgG TrueBlot HRP-conjugated secondary antibody (eBioscience) were used to capture immune complexes. Western immunoblotting was performed as previously described, using the antibodies listed above or anti-Smad4 antibodies (Calonge and Massagué, 1999). Band intensity was quantified with a Fujifilm LAS-3000 Imager and Image Gauge v4.0 software (Fujifilm).

ChIP

HaCaT cells were grown to 70% confluence, incubated in the presence or absence of TGFβ for 90 min, and subsequently crosslinked with 1% formaldehyde at room temperature for 15 min. ChIP was performed as described previously (Shang et al., 2000). The antibodies used were anti-Smad2/3 (Kretzschmar et al., 1999), anti-FoxO3 (Santa Cruz [H-144]), anti-Flag M2 (Sigma), anti-Myc (9E10, Santa Cruz), and anti-C/EBPβ (C-19, Santa Cruz). A 308 bp segment of the distal region of the *p15INK4b* promoter (nucleotides −547 to −239) was amplified with the following primers: 5′-TATGGTTGACTA ATTCAAACA-3′ (sense) and 5′-AATATTTTGGGAATGTTCACCA-3′ (antisense). In the proximal region of the *p15INK4b* promoter (nucleotides −177 to 161), a 338 bp segment was amplified with the following primers: 5′-*AGT CTCTGGCGCATGCGTCCTA*-3′ (sense) and 5′-*TTAGCTCCGGGCTTTTCCT GGC*-3′ (antisense). A region of the *c-MYC* promoter (nucleotides −206 to +196) harboring the TGFβ inhibitory element (TIE) was amplified with the

following primers: 5′-CTTTATAATGCGAGGGTCTGGAGC-3′ and 5′-GCTATGGGCAAAGTTTCGTGGATG-3′. As a negative control, a 166 bp region of the *β-actin* promoter (nucleotides 29 to 195) was amplified with the following primers: 5′-TCGAGCCATAAAAGGCAACTT-3′ (sense) and 5′-AAACTCTCCCTCCTCCTCTTCC-3′ (antisense).

Retroviral infection

H29 packaging cell lines were transfected with the pRetroSuper, pBabe-Puro, or pQCXI (Clontech) retroviral constructs using Lipofectamine following the manufacturer's instructions (Invitrogen). Viral supernatants were harvested 48 hr posttransfection, filtered (0.45 μM), and then concentrated at 18,000 rpm during 2 hr and used for overnight infections of HaCaT cells or EpCAM+ pleural effusion cells in the presence of 8 μg/ml of polybrene. Cells were then recovered for 24 hr with fresh media and then selected by 5 μg/ml puromycin for 24 hr.

Cell proliferation assays

A total of 10^5 cells/well were plated and treated with or without daily additions of TGFβ (100 pM). Cell numbers were recorded in triplicate over a 4 day period. ^{125}I-deoxyuridine incorporation assays were performed as described previously (Carcamo et al., 1995).

Quantitative real-time PCR assays

cDNA was synthesized from 1 μg of purified RNA using the SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen following the manufacturer's protocol. qRT-PCR was performed using a 7900HT instrument (Applied Biosystems). All reactions were performed in a volume of 10 μl containing 1 μl cDNA template (20 ng), 0.1 μM primers, and 5 μl of the SYBR Green I Master Mix (Applied Biosystems). Each sample was analyzed in quadruplicate. A no-template control was included for each primer set used. PCR cycling parameters were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, 60°C for 1 min. Data analysis was done using the comparative C_T method in software SDS2.2.2 (Applied Biosystems).

Animal studies

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Nude-beige (NIH-bg-nu-xidBR) (NCI) mice 4–6 weeks old were used for all xenografting studies. For mammary fat pad tumor assays, cells were harvested by trypsinization, washed twice in PBS, and counted. Cells were then resuspended (10^6 cells/ml) in a 50:50 solution of PBS and Matrigel. Mice were anesthetized, a small incision was made to reveal the mammary gland, and 10^5 cells were injected directly into the mammary fat pad. The incision was closed with wound clips, and primary tumor outgrowth was monitored weekly by bioluminescent imaging.

Bioluminescent imaging and analysis

Mice were anesthetized and injected retro-orbitally with 1.5 mg of d-luciferin (15 mg/ml in PBS). Imaging was completed between 2 and 5 min after injection with a Xenogen IVIS system coupled to Living Image acquisition and analysis software (Xenogen). For BLI plots, photon flux was calculated for each mouse by using a circle region of interest encompassing the mammary fat pad of the mouse. This value was scaled to a comparable background value (from a luciferin-injected mouse with no tumor cells).

Primers used for qRT-PCR

Primers were as follows: 18S, forward, CTCAACACGGGAAACCTCAC; 18S, reverse, CGCTCCACCAACTAAGAACG; CDC42EP3, forward, AGACTCGG CTGGATCTGC; CDC42EP3, reverse, GACCACAACCAGGACAAACC; CDK N1A, forward, CCGAGGCACTCAGAGGAG; CDKN1A, reverse, AGCTGCTC GCTGTCCACT; CDKN2B, forward, CAACGGAGTCAACCGTTTC; CDKN2B, reverse, GGTGAGAGTGGCAGGGTCT; CTGF, forward, CTGCAGGCTAGA GAAGCAGAG; CTGF, reverse, GATGCACTTTTTGCCCTTCT; GADD45A, forward, GCCAAGCTGCTCAACGTC; GADD45A, reverse, CTGGATCAGGG TGAAGTGG; GADD45B, forward, AGTCGGCCAAGTTGATGAAT; GADD45B, reverse, CCTCCTCCTCCTCGTCAAT; IER3, forward, CCGCAGGGTTCTC TACCC; IER3, reverse, GGATCTGGCAGAAGACGATG; JAG1, forward, TGC CAAGTGCCAGGAAGT; JAG1, reverse, GCCCCATCTGGTATCACACT; LEMD3, forward, AGGAATGAGAGGGACAGGAGT; LEMD3, reverse, TGTTTCACCA AAGGGGTTTT; OVOL1, forward, ACAGACCCCCAGAGCAGAG; OVOL1, reverse, GACTGTCCCCAAGGGTCAC; PAI, forward, AAGGCACCTCTGAGAA CTTCA; PAI, reverse, CCCAGGACTAGGCAGGTG; SGK, forward, CTGAG CTTATGAATGCCAACC; SGK, reverse, GCCAAGGTTGATTTGCTGAG.

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