ATF3, an adaptive-response gene, enhances TGFβ signaling and cancer-initiating cell features in breast cancer cells

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Summary

The activating transcription factor 3 (*ATF3*) gene is induced by a variety of signals, including many of those encountered by cancer cells. We present evidence that ATF3 is induced by TGFβ in the MCF10CA1a breast cancer cells and plays an integral role for TGFβ to upregulate its target genes snail, slug and twist, and to enhance cell motility. Furthermore, ATF3 upregulates the expression of the *TGFb* gene itself, forming a positive-feedback loop for TGFβ signaling. Functionally, ectopic expression of ATF3 leads to morphological changes and alterations of markers consistent with epithelial-to-mesenchymal transition (EMT). It also leads to features associated with breast-cancer-initiating cells: increased CD24^{low}–CD44^{high} population of cells, mammosphere formation and tumorigenesis. Conversely, knockdown of ATF3 reduces EMT, CD24^{low}–CD44^{high} cells and mammosphere formation. Importantly, knocking down twist, a downstream target, reduces the ability of ATF3 to enhance mammosphere formation, indicating the functional significance of twist in ATF3 action. To our knowledge, this is the first report demonstrating the ability of ATF3 to enhance breast cancer-initiating cell features and to feedback on TGFβ. Because *ATF3* is an adaptive-response gene and is induced by various stromal signals, these findings have significant implications for how the tumor microenvironment might affect cancer development.

Key words: ATF3, Cancer-initiating cells, Stress response

Introduction

ATF3 is a member of the ATF/CREB family of transcription factors, which share the basic region leucine zipper DNA binding motif and bind to the ATF/CRE consensus sequence TGACGTCA (for reviews, see Hai et al., 1999; Hai and Hartman, 2001; De Cesare and Sassone-Corsi, 2000; Sassone-Corsi et al., 1990). Overwhelming evidence indicates that ATF3 gene expression is upregulated by a variety of stress signals, including those encountered by cancer cells during their development. Examples include carcinogens, DNA damage, hypoxia and anoxia (Ameri et al., 2007) (for reviews, see Hai et al., 1999; Hai and Hartman, 2001). However, ATF3 is also induced by signals that do not fit the conventional definition of stress signals, such as adipokines and S-phase progression (for reviews, see Hai, 2006; Hai et al., 2006). Thus, *ATF3* can be viewed as an adaptive-response gene that participates in cellular processes to adapt to extra- and/or intra-cellular changes. Borrowing the concept from the network theory, we propose that ATF3 functions as a hub in the biological network that allows cells to respond to signals perturbing homeostasis.

Recently, we demonstrated that ATF3 has a dichotomous role in breast cancer cells in a context-dependent manner (Yin et al., 2008). On the one hand, it enhances stress-induced apoptosis in the MCF10A cells, an immortalized but untransformed breast cancer cell line. On the other hand, it protects the MCF10CA1a cells, a malignant derivative of MCF10A, from stress-induced cell cycle arrest and enhances its cell motility. These results provide a potential explanation to the conflicting literature that ATF3 can be either a tumor suppressor or an oncogene in xenograft injection models using different cell lines (Lu et al., 2006; Bottone et al., 2005; Ishiguro and Nagawa, 2000; Bandyopadhyay et al., 2006). Because MCF10A and MCF10CA1a are isogenic cell lines with the same genetic backgrounds except the genetic and/or epigenetic alterations incurred during malignant transformation, it indicates that the degree of malignancy of the cells enables ATF3 to have opposite functions. This is reminiscent of the TGFβ dichotomy, a phenomenon that TGFβ induces apoptosis or cell cycle arrest in normal or less-transformed cells but increases cell motility and metastasis in malignant cells (for reviews, see Massague, 2000; Derynck et al., 2001; Roberts and Wakefield, 2003). This parallel is intriguing in light of a previous report indicating a direct link between ATF3 and TGFβ. Massagué and colleagues demonstrated that ATF3 is induced by TGFβ in MCF10A, HaCaT (spontaneously immortalized skin keratinocytes) and HPL1 (SV40 T antigen transformed lung epithelia) cells. Upon induction, ATF3 interacts with SMAD3 and plays an integral role in the ability of TGFβ to repress its target gene *ID1* (Kang et al., 2003). All three cell lines

used in that study (MCF10A, HaCaT and HPL1) are non-malignant and TGFβ plays a cytostatic role. One important question is whether ATF3 also plays an integral role for TGFβ signaling in malignant cells, where TGFβ enhances cell motility and metastasis.

In this report, we present evidence that ATF3 is induced by TGFβ in the malignant breast cancer cells and plays an integral role for TGFβ to upregulate cell motility. Importantly, we found that ATF3 upregulates the expression of the *TGFb* gene itself, forming a positive-feedback loop. Functionally, ATF3 enhances epithelial-to-mesenchymal transition (EMT) and cancer-initiating cell features. Because ATF3 is induced by many stromal factors, our findings have significant implications on how tumor microenvironments might affect cancer development (see Discussion).

Results

ATF3 plays an integral role in the ability of TGFβ to regulate genes in the cell motility program

Previously, we identified several ATF3 target genes in the malignant breast cancer cells MCF10CA1a: twist, snail, slug, fibronectin (*FN*), plasminogen activator inhibitor 1 (*PAI1*), urokinase plasminogen activator (*uPA*), caveolin 1 (*CAV1*), type IV collagen alpha 2 (COL4A2) and secretory leukocyte peptidase inhibitor (*SLPI*) (Yin et al., 2008). All these genes are also known to be TGFβ-SMAD target genes. Intriguingly, their corresponding promoters contain close-by SMAD binding sites (SBEs) and ATF/CRE-like sites (supplementary material Fig. S1), suggesting that these promoters might be co-regulated by SMADs and ATF3. Previously, we showed that ATF3 is expressed at a low level in the MCF10CA1a cells and is further induced by TGFβ (Yin et al., 2008). A time-course analysis confirmed the induction and indicated that the induction persists from 45 minutes to at least 3 hours after TGFβ treatment (supplementary material Fig. S2). Interestingly, individually inhibiting some of the pathways activated by TGFβ (SMAD2/3, Akt, p38, ERK and JNK) using inhibitors or siRNAs did not significantly reduce TGFβ-induced ATF3 expression (supplementary material Fig. S3), suggesting that TGFβ induces ATF3 by multiple pathways. This is consistent with the literature that, although SMAD2/3 are the central mediators for TGFβ, other mediators also have important roles (Moustakas and Heldin, 2005; Moustakas and Heldin, 2008).

We then asked whether ATF3 is required for TGFβ to induce the above target genes. We focused on twist, snail, slug and FN because they were previously demonstrated to be direct target genes of ATF3, as evidenced by the binding of ATF3 to their promoters and by the increased polymerase II occupancy on their coding region upon ATF3 expression using a chromatin immunoprecipitation (ChIP) assay (Yin et al., 2008). As shown by qRT-PCR, knockdown of ATF3 by siRNAs reduced the ability of TGFβ to induce twist, snail and slug, but control siRNAs did not (Fig. 1A). By contrast, ATF3 knockdown did not affect the induction of FN. Immunoblot analyses revealed consistent results in protein levels (Fig. 1B; Fig. 4B). Taken together, these results indicate that ATF3 is necessary for TGFβ to induce twist, snail and slug, but not FN. The reduction in twist, snail and slug induction underscores the importance of ATF3 for their induction. The result for FN is not surprising, as TGFβ probably induces its target genes via multiple transcription factors. As a complimentary approach, we examined the effects of ectopic expression of ATF3 and found that ATF3 expression by itself – in the absence of TGFβ treatment – increased the expression of all four genes at both mRNA and protein levels (Fig. 1C,D; Fig.
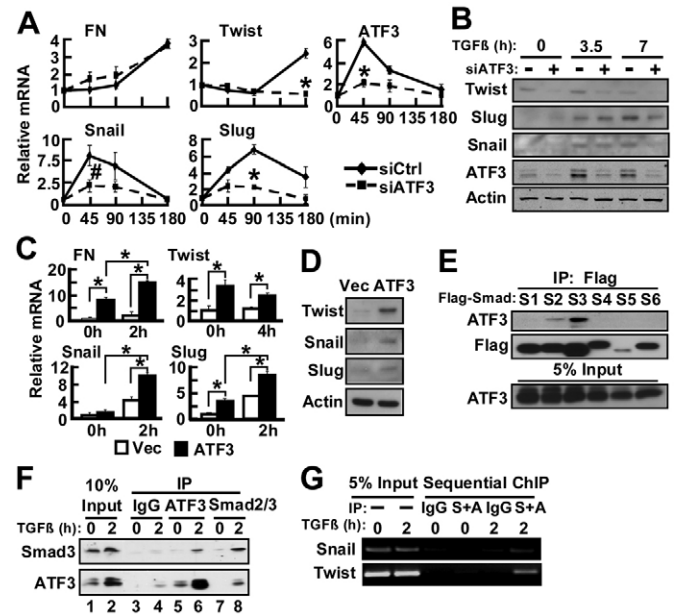

Fig. 1. ATF3 plays an integral role in the ability of TGFβ to regulate its target genes. (A) MCF10CA1a cells were transfected with control (siCtrl) or *ATF3* (si*ATF3*) siRNAs for 72 hours before TGFβ treatment for the indicated times. The mRNA levels of the indicated genes were determined by qRT-PCR and standardized against β-actin mRNA. The standardized level for each gene at time 0 was arbitrarily defined as 1. Shown is the average of three independent experiments (*$P<0.05$; #$P<0.08$). **(B)** Same as A, except that immunoblot for proteins using the indicated antibodies was shown. –, control siRNA; +, si*ATF3*. A representative of two independent experiments is shown. **(C)** MCF10CA1a cells with ectopic expression of ATF3 or the control vector (Vec) were treated for the indicated time or untreated (0h) with TGFβ and analyzed by qRT-PCR as in A. *$P<0.05$. **(D)** MCF10CA1a cells with ectopic expression of ATF3 or the control vector (Vec) were analyzed by immunoblot using the indicated antibodies. A representative of two independent experiments is shown. **(E)** COS-1 cells were transfected with DNAs to express *ATF3* and the indicated SMAD proteins (Flag-SMADs) followed by co-immunoprecipitation analyses (top). IP, immunoprecipitation. Five percent input is shown (bottom). **(F)** MCF10CA1a cells with (2h) or without (0h) TGFβ treatment were analyzed by co-immunoprecipitation as indicated. **(G)** MCF10CA1a cells with (2h) or without (0h) TGFβ treatment were analyzed by sequential ChIP using anti-SMAD2/3 antibody (S) followed by anti-ATF3 antibody (A) for immunoprecipitation. Rabbit IgG was used as a control.

4B). In the presence of TGFβ induction, ATF3 further increased the expression of FN, snail and slug, but not twist (Fig. 1C). This result is reproducible in multiple experiments at various time-points (2, 3, 4 and 6 hours after TGFβ treatment; data not shown). Thus, ATF3 is sufficient to induce twist expression and is necessary for TGFβ to induce Twist (panel A). However, it does not further enhance the ability of TGFβ to induce twist. The potential significance of this observation is not clear.

Because ATF3 was demonstrated to interact with SMAD3 (Kang et al., 2003), we examined the interactions between ATF3 and various SMAD proteins (SMADs 1–6). As shown by co-immunoprecipitation assay, exogenously expressed ATF3 interacted with Flag-tagged SMAD2 and SMAD3, but not other SMADs (Fig. 1E). The result for SMAD5 is not conclusive because SMAD5 was expressed at a low level in repeated experiments. Our results are slightly different from those reported by Kang et al. in that they

did not detect the interaction between ATF3 and SMAD2 (Kang et al., 2003). This could be owing to the relatively weak interaction between them. We also examined the interactions between the endogenous ATF3 and SMAD2/3 in the MCF10CA1a cells, using TGFβ to increase the levels of ATF3. Fig. 1F shows that immunoprecipitation of ATF3 brought down SMAD2/3 (lane 6) and vice versa (lane 8). This interaction, combined with the closeness of their respective binding sites on the target promoters suggests that they might be recruited to the same protein complexes on the promoters. To test this possibility, we carried out sequential ChIP. We immuno-isolated the chromatin complexes using antibodies against SMAD2/3 in the first precipitation, eluted the complexes from the precipitates and subsequently immunoprecipitated them using an antibody against ATF3. Fig. 1G shows that DNA fragments of interest from the twist and snail promoters were isolated by this method, suggesting that ATF3 and SMAD2/3 are likely to be in the same protein complexes. The specificity of the signals was indicated by the lack of bands using IgG in the second immunoprecipitation and the lack of bands in cells without TGFβ treatment, a treatment that induces ATF3 expression.

ATF3 forms a positive-feedback loop for TGFβ

Feedback loops are often observed in biology as a means to either amplify the signal in the case of positive feedback or dampen the signal in the case of negative feedback. We asked whether ATF3, as a mediator of TGFβ signaling, has a feedback role in the pathway. qRT-PCR analysis showed that the *TGFb* mRNA level was higher in the ATF3-expressing MCF10CA1a cells than in the vector cells (Fig. 2A), and immunoblot showed an increase in the intracellular TGFβ protein levels (Fig. 2B). Although this, per se, does not necessarily mean transcriptional regulation, it indicates that increasing TGFβ expression is a downstream event of ATF3. TGFβ is produced as a precursor and kept in a latent complex; its bioavailability is tightly regulated by multiple processes involving proteases and integrins (for reviews, see Barcellos-Hoff, 1996; Jenkins, 2008; Moustakas and Heldin, 2009). Among them, the $\alpha_v\beta6$ integrin heterodimer is of particular interest in the context of ATF3 biology because the β6 (*ITGB6*) gene is stress-inducible (Breuss et al., 1995; Breuss et al., 1993). We tested whether ATF3 affects the expression of this integrin heterodimer. As shown in Fig. 2A, the mRNA levels of both α_v and β6 are increased in the ATF3-expressing cells. To test whether ATF3 expression increases bioavailable TGFβ, we used a TGFβ reporter cell developed by Rifkin and colleagues, where a mink lung epithelial cell line was engineered to harbor a luciferase reporter driven by a fragment of the *PAI1* promoter. This fragment responds well to TGFβ but not other known inducers of PAI1 expression, and the reporter cell line was demonstrated to detect TGFβ bioactivity in a highly specific, sensitive and dose-dependent manner (Abe et al., 1994). Fig. 2C shows that conditioned media from ATF3-expressing MCF10CA1a cells elicited higher luciferase activity than that from the vector cells. Consistent with this result, MCF10CA1a cells ectopically expressing ATF3 have higher phospho-SMAD3 than the control cells (Fig. 2B), suggesting an increase in TGFβ signaling.

ATF3 enhances cell motility and features of epithelial-to-mesenchymal transition

Twist, snail and slug are important regulatory genes of cell motility. Thus, we tested whether ATF3 knockdown could reduce TGFβ-induced cell motility. To this end, we generated stable cell lines
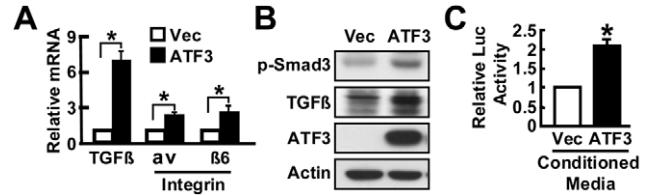

Fig. 2. ATF3 positively feeds back on TGFβ. (**A**) MCF10CA1a cells ectopically expressing ATF3 or the vector (Vec) were analyzed by qRT-PCR for the indicated mRNAs. Standardized signals in the control cells were arbitrarily defined as 1 and the average of three independent experiments is shown (*$P<0.05$). (**B**) ATF3 or vector (Vec) cells were analyzed by immunoblot for the indicated proteins. p-SMAD3, phospho-SMAD3; TGFβ, intracellular TGFβ. Shown is the representative of three experiments. (**C**) Conditioned medium was collected from MCF10CA1a cells ectopically expressing ATF3 or vector only (Vec) and incubated with the TGFβ reporter cells. The reporter activity was determined by luciferase assay and the number from the conditioned medium derived from Vec cells was arbitrarily defined as 1. The average of three independent experiments is shown (*$P<0.05$).

expressing an shRNA to knockdown ATF3 as detailed in the Material and Methods. Immunoblot for ATF3 showed efficient, albeit incomplete, knockdown of ATF3 (Fig. 3B). The ATF3 knockdown cells had ~25% reduction in cell motility compared with the control knockdown cells under the condition of TGFβ induction (Fig. 3A, bars 3 and 4; $P<0.05$). To complement the shRNA experiments, we used an siRNA pool to transiently inhibit ATF3 expression and obtained similar results (supplementary material Fig. S4A). However, the extent of reduction in motility is only ~10% ($P<0.05$). This subtle effect might be due to the inefficient knockdown of ATF3 by the transient method (supplementary material Fig. S4B). Because the shRNA and siRNA
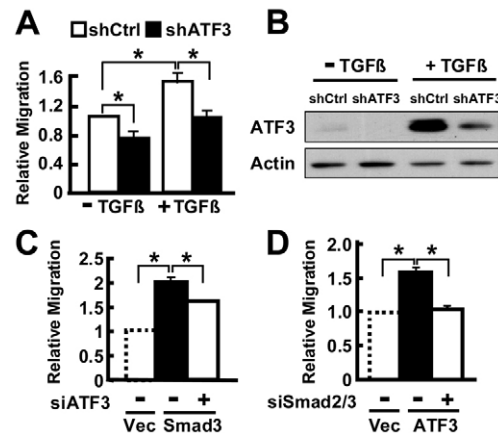

Fig. 3. ATF3 promotes cell motility. (**A,B**) MCF10CA1a cells stably expressing control (shCtrl) or ATF3 (sh*ATF3*) shRNA were analyzed by the Boyden chamber migration assay in the presence or absence of 2.5 ng/ml TGFβ (A; *$P<0.05$, derived from three experiments). ATF3 protein levels were determined by immunoblot (B; a representative of three experiments). (**C**) MCF10CA1a cells ectopically expressing SMAD3 were transfected with control siRNAs (–) or siRNAs to knockdown ATF3 (+) for 72 hours, followed by a cell motility assay. Migration of cells without ectopic expression of SMAD3 was arbitrarily defined as 1 (*$P<0.05$). (**D**) As in C, except that ATF3-expressing cells were used and SMAD2/3 was knocked down (*$P<0.05$).

sequences target different regions of ATF3, these knockdown results are probably not due to an off-target effect.

Previously, Tian et al. showed that MCF10CA1a cells ectopically expressing SMAD3 have higher cell motilities than the corresponding vector control cells (Tian et al., 2003). We asked whether ATF3 knockdown would dampen the effect of SMAD3. Fig. 3C shows that it did (compare bar 3 with bar 2; $P<0.05$). Conversely, SMAD2/3 knockdown dampened the motility of cells ectopically expressing ATF3 (Fig. 3D; $P<0.05$). These results, in conjunction with those presented in Fig. 1, indicate that ATF3 is an integral component of the TGFβ-regulated cell motility program. It is required for TGFβ to upregulate its target genes twist, snail and slug – important regulators of cell motility. In addition, it interacts with SMAD2/3 and is likely to be in the same complexes as SMAD2/3 on the twist and snail promoters. Functionally, it is partially required for TGFβ to increase cell motility.

During the course of our investigation, we noticed that MCF10CA1a cells ectopically expressing ATF3 are more elongated and scattered than the vector control cells, which are cuboidal and clustered (Fig. 4A). These morphological changes are classic features of EMT (Guarino, 2007). Consistently, immunoblot analyses showed molecular changes commonly associated with EMT in ATF3-expressing cells: decreased E-cadherin but increased N-cadherin, vimentin and FN expression (Fig. 4B). Because the MCF10CA1a cells express a basal level of endogenous ATF3 (Yin et al., 2008) (Fig. 4B), we asked whether reducing ATF3 expression would have an opposite effect. Fig. 4B shows that ATF3 knockdown reduced EMT features as indicated by the molecular markers. To test whether this phenomenon is limited to the MCF10CA1a cells, we examined MCF7 cells. As the endogenous level of ATF3 is undetectable in MCF7 cells, we generated a stable cell line expressing ATF3 without carrying out the knockdown experiments. As shown in Fig. 4C,D, ectopic expression of ATF3 resulted in similar morphological changes – from cuboidal to spindle – and a similar alteration in EMT markers: increase N-cadherin, vimentin and FN, but decreased E-cadherin. Taken together, ATF3 is a positive regulator of EMT. Significantly, it can do so even in the absence of exogenous TGFβ treatment. Because ATF3 is induced in the MCF10CA1a breast cancer cells by TNFα, IL1β and hypoxia (supplementary material Fig. S2) – signals that are commonly present in the tumor microenvironments (Knowles and Harris, 2007; Fox et al., 2007) – these results have significant implications. They suggest a possibility that various stromal signals, by inducing ATF3, might enhance EMT (see Discussion).

ATF3 enhances breast cancer-initiating cell features in vitro

Cells that have undergone EMT were recently demonstrated to display enhanced cancer-initiating cell features (Mani et al., 2008; Morel et al., 2008) (for a review, see Polyak and Weinberg, 2009). This prompted us to examine whether ATF3 expression affects the cancer-initiating cell features in the MCF10CA1a cells. As shown by fluorescence-activated cell sorting (FACS) analysis, MCF10CA1a cells with ectopic expression of ATF3 had an increased percentage of cells with the surface markers CD24^{low} and CD44^{high}, a feature associated with human breast cancer-initiating cells (Al-Hajj et al., 2003). Fig. 5A shows representative FACS images and Fig. 5B shows the quantitation from eight repeated experiments (2.3±0.46-fold increase; $P<0.05$). qRT-PCR analyses showed reduced CD24 expression in the ATF3-expressing cells (supplementary material Fig. S5), providing a potential explanation for the increase of the CD24^{low} population. We also examined the expression of a few other markers that have been shown to associate with breast cancer-initiating cells (Korkaya et al., 2008); among the markers examined, only GLI1 showed higher
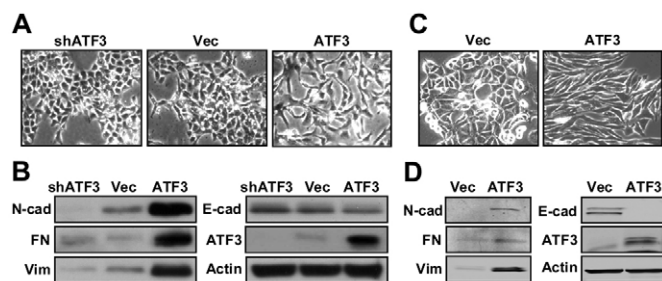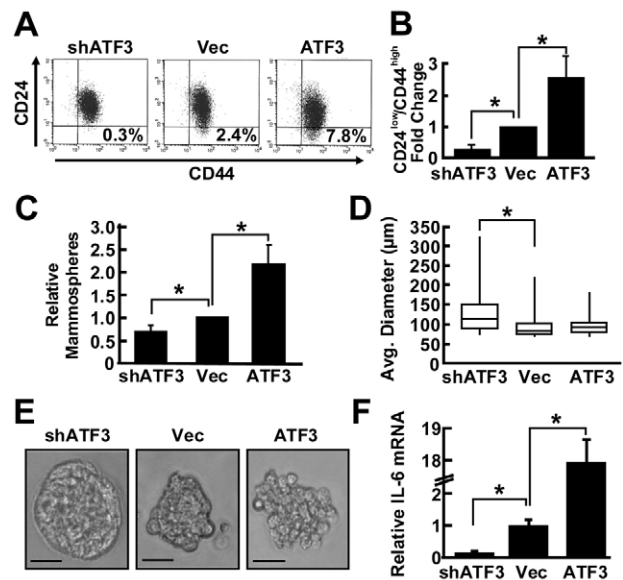

Fig. 4. ATF3 enhances EMT. (A) Stable derivatives of MCF10CA1a cells with ATF3 knockdown (sh*ATF3*), ectopic expression of ATF3 or vector control (Vec) were photographed under a light microscope (at ×200 magnification). (B) Cells as indicated in A were analyzed by immunoblot for the indicated proteins. N-cad, N-cadherin; E-cad, E-cadherin; FN, fibronectin; Vim, vimentin. (C) Stable derivatives of MCF7 cells with ectopic expression of ATF3 or vector control (Vec) were photographed under a light microscope (at ×200 magnification). (D) Cells as indicated in C were analyzed by immunoblot for the indicated proteins. All results in this figure are representatives of at least three independent experiments.

Fig. 5. ATF3 enhances cancer-initiating cell features. (A,B) Stable derivatives of MCF10CA1a cells with ATF3 knockdown (sh*ATF3*), ectopic expression of ATF3 or vector control (Vec) were analyzed by FACS for the CD24 and CD44 surface markers. A shows representative images and B the quantitation from eight repeated experiments (*$P<0.05$). Numbers in A indicate the percentage of the CD24^{low}–CD44^{high} population. (C–E) The indicated cells were seeded to form mammospheres and analyzed for mammosphere forming efficiency (C; *$P<0.05$, from four experiments), size (D; *$P<0.05$, from four experiments) and representative phase-contrast images (E). For C, only mammospheres with a diameter ≥70 μm were counted and the number of mammospheres derived from vector control cells was arbitrarily defined as 1 (*$P<0.05$, from four experiments). Scale bars: 40 μm in E. (F) Mammospheres derived from the indicated cells were analyzed for their *IL6* mRNA levels by qRT-PCR. Standardized signal from vector control cells was arbitrarily defined as 1 (*$P<0.05$, from four experiments).

expression in the ATF3-expressing cells than in the vector control cells (supplementary material Fig. S5). We then examined the ability of the cells to form mammospheres under non-adherent conditions, another feature of breast cancer-initiating cells (Dontu et al., 2003; Liao et al., 2007). The ATF3-expressing cells formed more mammospheres (Fig. 5C), although the size of the mammosphere did not change to any appreciable degree (Fig. 5D). As an opposite approach, we used stable cells with ATF3 knockdown by the shRNA approach (described above). As shown in Fig. 5A–C, knocking down the endogenous ATF3 levels in the MCF10CA1a cells reduced cancer-initiating cell features, as evidenced by the reduction in their mammosphere formation efficiency and the percentage of CD24^{low} and CD44^{high} cells. To complement the shRNA knockdown method, we repeated the experiments using siRNAs and found similar results: reduction in mammosphere formation and CD24^{low}–CD44^{high} population of cells (supplementary material Fig. S6).

We observed that the MCF10CA1a mammospheres lack clear and complete borders, a morphology similar to that observed in the mammospheres formed by the malignant MDA-MB-231 cells (Grimshaw et al., 2008). Ectopic expression of ATF3 further reduced the integrity of the border but knockdown of ATF3 dramatically increased it. Fig. 5E shows representative phase-contrast images of these mammospheres. Although the biological significance of this morphological change is not clear, it might be associated with the aggressiveness of the mammospheres. Recently, Sansone et al. demonstrated that mammospheres derived from invasive breast carcinoma expressed higher levels of IL6 than those from matched, non-neoplastic mammary glands (Sansone et al., 2007). Interestingly, mammospheres from ATF3-expressing cells had higher IL6 expression than that from the vector control cells; conversely, mammospheres from ATF3-knockdown cells had lower IL6 expression (Fig. 5F). Therefore, ATF3 expression in the mammospheres correlated with the expression of IL6 – a marker for aggressiveness. We note that ATF3 also increased the ability of MCF10A cells, the untransformed parental cells of MCF10CA1a (Santner et al., 2001), to form mammospheres (supplementary material Fig. S7A). However, it had an opposite effect on their morphology. As shown in Fig. S7B, ATF3 increased, rather than decreased, the integrity of the mammosphere border. This opposite effect of ATF3 is reminiscent of its dichotomous function in this pair of isogenic cells that we reported previously (Yin et al., 2008): ATF3 enhances the ability of stress signals to induce apoptosis in the untransformed MCF10A cells but protects the malignant MCF10CA1a cells from stress-induced deleterious effects.

Twist plays a functionally important role in the ability of ATF3 to enhance mammosphere formation

To test whether ATF3 enhances mammosphere formation, at least in part, by upregulating its target genes, we examined the effect of knocking down twist in the MCF10CA1a cells ectopically expressing ATF3. As shown in Fig. 6, twist knockdown reduced mammosphere formation and the population of CD24^{low}–CD44^{high} cells. Although this result by itself does not necessarily indicate any link between ATF3 and twist, in conjunction with the above results that ATF3 upregulates twist expression, it indicates that Twist is functionally important for ATF3 action.

ATF3 enhances breast cancer cell tumorigenicity in vivo

To test whether ectopic expression of ATF3 increases tumorigenicity, we injected the cells into nude mice as detailed in
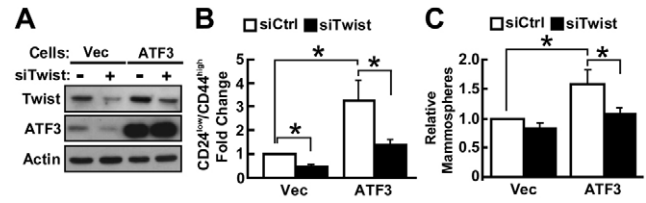

Fig. 6. Knockdown of twist reduces the ability of ATF3 to enhance cancer-initiating cell features. Stable derivatives of MCF10CA1a cells with ectopic expression of ATF3 or vector control (Vec) were transfected with control (siCtrl) or twist (siTwist) siRNAs for 72 hours before being analyzed by immunoblot using the indicated antibodies (**A**), by FACS for the CD24 and CD44 surface markers (**B**) and for mammosphere formation (**C**). Only mammospheres with a diameter ≥70 μm were counted. A shows a representative image and B and C show the quantitation from three independent experiments (*$P<0.05$). For both panels, the number derived from vector control cells with control siRNA knockdown was arbitrarily defined as 1.

the Materials and Methods. As shown in Table 1, six out of six mice injected with 10^4 ATF3-expressing cells developed tumors but only one out six mice receiving the vector control cells did. At 10^3 cells, the ATF3-expressing cells gave rise to tumors in two out of six mice but the control cells failed to do so in any of the six mice injected (Table 1). At 10^5 cells, no difference was observed between the vector and ATF3-expressing cells (Table 1). Presumably, this is because the number of cells injected was saturating or the number of mice used was under powered, or both. Taken together, ATF3 increased the tumor-initiating frequency, again indicating that ATF3 increases cancer-initiating cell features.

Discussion

The human *ATF3* gene is located in the 1q amplicon, the second most frequently amplified chromosomal region in human solid tumors (Rooney et al., 1999). Previously, we demonstrated that the *ATF3* gene is amplified in ~80% of the human breast tumors examined (total 48) and its expression elevated in ~50% of them (Yin et al., 2008). Because no mutations were found in the open reading frame of the *ATF3* gene (Yin et al., 2008), its upregulation of expression suggests an oncogenic role for ATF3. Using immunohistochemistry, MacLeod and colleagues showed that ATF3 is upregulated in many human breast tumors (Wang et al., 2008). Thus, they also suggested that ATF3 acts as an oncogene in breast carcinoma. Thus far, the mechanisms for the oncogenic roles of ATF3 in breast cancer are not well understood. Previously, we showed that ATF3 promotes cell motility and protects malignant breast cancer cells from stress-induced cell cycle arrest in vitro (Yin et al., 2008). In this report, we present three lines of evidence that provide insights to the oncogenic roles of ATF3: (1) it mediates

Table 1. Tumor incidence of the indicated cancer cells injected into nude mice in limiting dilutions

	Tumor incidence per number of mice		
Cells injected	1×10^3	1×10^4	1×10^5
Vec	0/6	1/6	3/3
ATF3	2/6	6/6	3/3

ATF3, MCF10CA1a cells ectopically expressing ATF3; Vec, MCF10CA1a cells expressing the vector control.

TGFβ signaling; (2) it positively feeds back on TGFβ; and (3) it enhances EMT, cancer-initiating cell features and tumorigenesis.

Although ATF3 was shown to mediate TGFβ-induced downregulation of *ID1* gene expression (Kang et al., 2003), the current study differs from the previous study in a key aspect: we used malignant breast cancer cells, where TGFβ is pro-metastatic; the previous study used non-malignant cells, where TGFβ is cytostatic. Thus, the interplay between ATF3 and TGFβ-SMAD is not limited to the regulation of *ID1*, a cell cycle regulatory gene. Instead, it extends to the regulation of other TGFβ target genes, such as twist, snail and slug, which are cell motility regulators. In fact, this phenomenon is likely to be broadly applicable. Using a bioinformatics approach (detailed in the Materials and Methods), we scanned 29,895 RefSeq-annotated genes for the ATF and SMAD consensus binding sequences and found 1846 co-occurrences within 100 bp, corresponding to 1823 unique genes. Among them, two gene functional annotations were enriched ($P<0.01$): calmodulin binding (*CAMK1*, *MYO5C*, *MYO9B*, *PCNT*, *TRPV6*, *KCNN3*, *SLC8A3*) and regulation of the cell cycle (*CDK9*, *TP53*, *MDM2*, *MYC*, *STAT5B*, *GADD45A*, *GADD45B*, *UBA3*, *ZBTB17*, *F2*, *SLC12A6*, *PTMS*, HBP1, *LIN9*, *NME1*). See supplementary material Table S1 for annotation. Clearly, experimental tests are required to determine whether these candidates are indeed target genes for ATF3 and SMADs. Nevertheless, this result supports the idea that many promoters might be co-regulated by SMADs and ATF3 (and perhaps other ATF proteins). This supposition is consistent with a well-documented phenomenon that SMAD2/3 bind to their cognate sites with low affinity and require co-transcription factors to increase their binding (for reviews, see Massague et al., 2000; Moustakas and Heldin, 2009). The sequential ChIP data in Fig. 1G suggest that ATF3 and SMAD2/3 are recruited to the same protein complexes on the twist and snail promoters. Because knockdown of ATF3 reduced the ability of TGFβ to upregulate these genes, we propose that ATF3 is an essential co-transcription factor for SMAD2/3 to activate these promoters.

One novel finding of our studies is that ATF3 potentially feeds back on TGFβ. ATF3 not only results in the upregulation of the *TGFb* gene itself, but also integrins $α_v$ and β6 (Fig. 2A) – two genes involved in the activation of TGFβ (for reviews, see Barcellos-Hoff, 1996; Jenkins, 2008). Previously, we showed that ATF3 upregulates the expression of uPA and PAI1 (Yin et al., 2008), two regulators in the proteolytic cascade to produce soluble TGFβ (Gleizes et al., 1997). Although not a definitive proof, these results taken together provide a potential explanation for the higher TGFβ signaling in the ATF3-expressing cells than in the vector cells: an increase in the intracellular p-SMAD3 level and reporter activity (Fig. 2B,C). The possibility that ATF3 could activate the TGFβ pathway (directly or indirectly) has significant implications. Because ATF3 is induced by many stress signals, it might function as a nexus to allow other signals to turn on the TGFβ pathway. Clearly, this is only a speculation at this point. Whether any cross-activation occurs will depend on the cellular contexts and the other events initiated by the signals (see more below).

Another novel finding of our studies is that ATF3 enhances cancer-initiating cell features, as indicated by its ability to increase the population of CD24^{low}–CD44^{high} cells, the mammosphere-forming ability, and the tumor-initiating frequency. Similar to the normal stem cells, the cancer-initiating cells can self-renew and differentiate. The cancer-initiating cell (or cancer stem cell) model posits that tumors contain a small fraction of cancer-initiating cells, which are the cells responsible for giving rise to the bulk of the tumor mass (Marotta and Polyak, 2009; Tan et al., 2006; Shipitsin and Polyak, 2008). Owing to the nature of ATF3 inducibility, our finding has significant implications. ATF3 is induced by many extracellular signals. In addition to TGFβ, ATF3 is induced in the MCF10CA1a breast cancer cells by TNFα, IL1β and hypoxia (supplementary material Fig. S2), signals that are commonly present in the tumor microenvironments (Knowles and Harris, 2007; Fox et al., 2007). Because ectopic expression of ATF3 in MCF10CA1a is sufficient to increase EMT, cancer-initiating cell features and tumorigenesis (Figs 4, 5; Table 1), this opens up the possibility that various stromal signals can exert their impact by inducing ATF3, a hub of the biological network, to promote cancer development, perhaps in part by cross-activating the TGFβ pathway. Clearly, whether a given tumor microenvironment can successfully increase cancer-initiating cell features would depend on the balance of various responses from the cancer epithelia to all the signals emanated from the environment. The concept that the stroma can regulate EMT and cancer-initiating cell features is not new (for reviews, see Moustakas and Heldin, 2007; Polyak and Weinberg, 2009; Ailles and Weissman, 2007). However, our results identified a specific adaptive-response gene, *ATF3*, as a potential nexus in the cancer epithelia through which the tumor microenvironments might exert their influence. Because cancer-initiating cells are potential therapeutic targets, our findings suggest that inhibition of ATF3 might have clinical relevance in cancer treatment.

Materials and Methods

Cell culture, treatments and antibodies
siRNA pools against specific target genes (Dharmacon, Lafayette, CO) were transfected into cells using DharmaFECT (Dharmacon). Seventy-two hours after transfection, cells were treated with 2.5 ng/ml TGFβ (R&D Systems, Minneapolis, MN) for the indicated times. Primary antibodies are from the following sources: anti-ATF3, anti-twist (Santa Cruz, Santa Cruz, CA), anti-snail, anti-slug, anti-E-cadherin, anti-SMAD3, anti-phospho-SMAD3, anti-ERK, anti-phospho-ERK, anti-ATF2, anti-phospho-ATF2, anti-Akt, anti-phospho-Akt, anti-TGFβ1 (Cell Signaling, Beverly, MA), anti-SMAD2/3 (BD Biosciences, San Jose, CA), anti-actin and anti-Flag (Sigma, St Louis, MO). The horseradish-peroxidase-linked secondary antibody is from Cell Signaling.

Generation of stable cells with ATF3 knockdown or ectopic expression of ATF3
The U6 promoter and shRNA targeting ATF3 were subcloned into the *Bam*HI site of the pBabe-Puro vector. The shRNA was designed to target a region of the *ATF3* exon E (5′-GGAACATTGCAGAGCTAAG-3′) and was previously demonstrated to knock down ATF3 expression in the pancreatic β cells (Li et al., 2007). MCF10CA1a cells were transduced with a retrovirus encoding ATF3 shRNA and selected with 2.5 μg/ml puromycin (InvivoGen, San Diego, CA) to generate stable cells. Clones were isolated by limited dilution and screened for knockdown efficiency by immunoblot. Stable cell lines with ectopic expression of ATF3 were generated by transducing the MCF10CA1a or MCF7 cells with retrovirus as described previously (Yin et al., 2008).

RNA isolation, quantitative reverse transcription coupled with polymerase chain reaction (qRT-PCR), immunoblot, co-immunoprecipitation, cell motility assay and sequential ChIP
All methods were carried out as described previously (Yin et al., 2008; Lu et al., 2006; Chen et al., 1996). For sequential ChIP, the protein-DNA complexes from the first immunoprecipitation were eluted with 10 mM DTT at 37°C for 30 minutes, diluted with sonication buffer by 40-fold and used for the second immunoprecipitation. See Table S2 in the supplementary material for the sequences of the primers used in this study.

The cell-based TGFβ reporter assay
The reporter assay was performed as described previously (Abe et al., 1994). Briefly, $5×10^4$ mink lung epithelial cells stably transfected with a reporter construct encoding −799 to +71 of the human plasminogen activator inhibitor 1 promoter fused to firefly luciferase were seeded into each well of a 96-well tissue culture plate. Conditioned medium prepared by a 24-hour incubation of ATF3-expressing or vector control cells in serum free medium, as described previously (Yin et al., 2008), was added to the

reporter cells. After an overnight incubation, the reporter activity was assayed using the Luciferase Assay System (Promega, Madison, WI).

FACS analysis

Cells were harvested, resuspended at the density of 10^6 cells/100 μl and incubated with antibodies against human CD44-FITC and CD24-PE or isotype controls (BD Biosciences) for 30 minutes on ice. Cells were washed three times with cold PBS containing 1% fetal bovine serum and analyzed on a BD FACSCalibur flow cytometer (BD Biosciences).

Primary mammosphere assay

Cells were harvested, washed twice with PBS and resuspended at a concentration of 10,000 cells/ml in mammosphere media: DMEM/F-12 supplemented with 20 ng/ml bFGF (Millipore, Billerica, MA), 20 ng/mL EGF (Millipore), 1× B27 (Invitrogen, Carlsbad, CA) and 4 μg/ml heparin (Sigma). Thirty thousand cells per well were seeded into ultra-low-attachment 6-well plates (Fisher Scientific, Pittsburgh, PA). After 7–10 days, mammospheres were imaged and quantified. Only mammospheres with a diameter >70 μm were counted.

Tumorigenicity assay

Six- to eight-week-old female athymic nude mice (Harlan Laboratories) were injected subcutaneously in the hind flank with 10^5, 10^4 or 10^3 cells diluted 1:4 in matrigel (BD Biosciences). Tumor incidence was monitored for 25 days following injection. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Ohio State University.

Promoter scanning

Promoter sequences spanning 1 kb upstream of the transcription start sites from 29,895 RefSeq-annotated genes were downloaded from the UCSC genome portal (http://hgdownload.cse.ucsc.edu) and scanned using the program Fuzznuc from the EMBOSS package (Mullan and Bleasby, 2002) for the *ATF* and *SMAD* consensus binding sequences within a sliding 100 bp window. The patterns used are the following: *SMAD*, AGACNC(C/G/T)C; *ATF*, TGACGTCA, (A/C/G)TGA(C/G)TCA and (A/C/C)T(G/T)(A/T/G)CNTC(A/T)(T/G/C). The lists of RefSeq identifiers with ATF/SMAD sequence co-occurrences were mapped to HUGO gene names and analyzed for overrepresented gene functions (that is, the functional enrichment analysis) using the NCI DAVID tool (Dennis et al., 2003) with the threshold for statistical significance at $P<0.01$.

Statistics

All quantitative data are expressed as mean ± s.d. and comparisons were made by the Student's *t*-test.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/20/3558/DC1

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