Regulation of the Anaphase-promoting Complex–Separase Cascade by Transforming Growth Factor- β Modulates Mitotic Progression in Bone Marrow Stromal Cells

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Alteration of the tumor microenvironment by aberrant stromal cells influences many aspects of cell biology, including differentiation of stem cells and tumor metastasis. The role of transforming growth factor (TGF)- β signaling in stromal cells of the tissue microenvironment is critical to both pathways. We examined murine marrow stromal cells with deletion of Smad3 and found that they have an altered cell cycle profile, with a higher fraction of cells in G2/M phase. Deletion of Smad3 significantly abrogates TGF- β signaling and suppresses phosphorylation of CDC27–anaphase-promoting complex (APC) during mitosis, thereby resulting in elevated cyclin-dependent kinase (CDK)1 activity via increased levels of cyclin B. Enhanced CDK1 activity due to deregulation of APC leads in turn to hyperphosphorylation of separase, impeding chromatid separation. A residue Ser1126Ala mutation in separase specifically abolished separase hyperphosphorylation in Smad3-deficient cells. The present results unveil a new function for the TGF- β pathway in the regulation of APC to mediate chromatid separation during mitosis.

INTRODUCTION

Transforming growth factor (TGF)- β , a pluripotent cytokine, plays an pivotal role in regulating many cellular events, including cell growth inhibition, differentiation, matrix production, apoptosis, and tumor metastasis (Derynck *et al.*, 2001; Roberts *et al.*, 2003; Siegel and Massague, 2003; Brier and Moses, 2006a). Deregulation of TGF- β has been implicated in enhancing progression of malignancy (Brier and Moses, 2006b; Gupta and Massague, 2006). Transduction of TGF- β signals from the cell surface to the nucleus is mediated by dimerization of type II and type I receptors followed by activation of a cascade of intracellular signal-transducing proteins called Smads (Massague and Wotton, 2000). The effects of TGF- β on cell lineage differentiation during development and in tumor cell growth during stages of tumorigenesis are variable (Siegel and Massague, 2003; Waite and Eng, 2003). The downstream TGF- β -activated effectors are quite different based on cell type and cellular status (Roberts *et al.*, 2003; Siegel and Massague, 2003). The prominent function of TGF- β in tumor suppression as well as cellular differentiation is through cell cycle regulation (Liu, 2006). Various TGF- β -regulated molecules have been identified with functions that principally govern G1 progression (Massague, 2004; Liu, 2006), whereas some mitotic players, including

cyclin B and CDC2, are also implicated as targets of TGF- β signaling during the cell cycle (Choi *et al.*, 1999; Liu *et al.*, 1999; Wan *et al.*, 2001). Recent studies addressing Smad3 function in cells of the hematopoietic system have revealed that knockout of Smad3 in mice results in a significant accumulation of a G2/M population of bone marrow stromal cells (BMSCs) (Epperly *et al.*, 2006). This observation suggested a potential novel regulatory mechanism for TGF- β in G2/M progression during the cell cycle. The mechanism of such a G2/M regulation by TGF- β signaling pathway is unknown.

In tumor development, mutated stromal cells can alter the tumor microenvironment and promote metastasis (Wiseman and Werb, 2002; De Wever and Mareel, 2003; Orimo *et al.*, 2005; Dong and Blobel, 2006; Roberts *et al.*, 2006). In the hematopoietic system, BMSCs are an integral component of the bone marrow that provides and responds to cytokines that are involved in directing cell lineage (Pittenger *et al.*, 1999). Deregulation of TGF- β signaling in the stroma affects immune cells involved in micro-immunosurveillance and thereby also can promote migration by tumor cells (De Wever and Mareel, 2003; Epperly *et al.*, 2005; Dong and Blobel, 2006). The expression of TGF- β in BMSCs contributes to the development of the hematopoietic cell lineages, including bone, cartilage, fat, tendon, and muscle (Pittenger *et al.*, 1999). Disruption of TGF- β signal transduction in BMSCs can lead to alteration of cell cycle progression, cell migration, and to radioresistance, which in turn can affect hematopoiesis as well as bone marrow-mediated tumorigenesis (Epperly *et al.*, 2005, 2006; Zhou *et al.*, 2005). Previous studies have focused extensively on how TGF- β signaling integrates

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with other signaling pathways to regulate cell lineage differentiation and cell migration and adhesion (Watanabe and Whitman, 1999; Massague and Chen, 2000; Waite and Eng, 2003). Little is known about the role of TGF- β in regulating cell cycle progression in BMSCs. The recent discovery that loss of TGF- β signaling abrogates BMSC G2/M progression suggests a new direction for studying TGF- β function. Understanding the molecular basis of how G2/M is regulated by TGF- β could unveil a novel mechanism by which TGF- β affects G2/M during the cell cycle and further enhance our knowledge of stromal cell biology.

To elucidate the mechanism by which G2/M is regulated by TGF- β signaling in BMSCs, we have integrated biochemical and immunocytochemical assays with an *in vivo* analysis. We demonstrate that the accumulation of cells at G2/M in Smad3^{-/-} cells is attributable to a failure of TGF- β -mediated mitotic regulation. In addition, we show that regulation of mitotic progression by TGF- β in BMSCs is through targeting the APC-separase pathway. Together, these results suggest that APC-dependent regulation of CDK1 activity, which in turn modulates chromatid separation, is a target for TGF- β signaling during mitosis.

MATERIALS AND METHODS

Cell Culture, Synchronization, Transfection, and Retroviral Infection

Wild-type and Smad3^{-/-} bone marrow stromal cell lines were described previously (Epperly *et al.*, 2006). Bone marrow stromal cells were cultured in α -minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA), 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Primary bone marrow stromal cells were grown in MyeloCult 5300 (Stem Cell Technologies, Vancouver, BC, Canada). To synchronize cells in mitosis, cells were grown in the presence of 0.4 mM thymidine (Sigma-Aldrich, St. Louis, MO) for 18 h, washed with phosphate-buffered saline (PBS), and grown in the fresh medium for 4 h, followed by 50 ng/ml nocodazole for 12 h and release (Wan *et al.*, 2001). To synchronize cells at the G1/S transition, cells were treated with thymidine twice and then released. For flow cytometric analysis, cells fixed in ethanol were washed and subsequently stained in PI buffer [10 μ g/ml propidium iodide, 5 μ g/ml RNase A [Sigma-Aldrich] in PBS) (Wan *et al.*, 2001). Transfection was done in Opti-MEM1 (Invitrogen) using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Infection/transduction of retrovirus carried Smad3 in Smad3^{-/-} cells was described previously (Liu *et al.*, 2000).

Mutagenesis

Mutants, including separase (S1073A/S1126A/S1305A/T1346A/S1501A/S1508A/S1545A/S1552A), separase (S1073A/S1305A/T1346A/S1501A/S1508A/S1545A/S1552A), and separase (S1126A), were engineered using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The presence of the desired substitutions and the absence of other mutations were confirmed by sequence.

Antibodies and Reagents

Immunoblotting analyses and immunoprecipitation were performed using the following antibodies: Cdh1 (Calbiochem, San Diego, CA), CDC2 (Santa Cruz Biotechnology, Santa Cruz, CA), Plk (Santa Cruz Biotechnology), BubR1 (Santa Cruz Biotechnology), NEK (Santa Cruz Biotechnology), securin (MBL International, Woburn, MA), cyclin B1 (Santa Cruz Biotechnology), APC2 (Santa Cruz Biotechnology), APC6 (Santa Cruz Biotechnology), separase (Santa Cruz Biotechnology; Novus Biologicals, Littleton, CO), tubulin (Calbiochem), and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit and anti-rat secondary antibody (Promega, Madison, WI). Monoclonal anti-TGF- β neutralizing antibody (R&D Systems, Minneapolis, MN) was used for TGF- β blocking experiment. Western blot analysis was performed using ECL detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Semiquantification of data was performed using densitometer.

Ubiquitylation Assay

Wild-type and Smad3 deletion cells were synchronized using nocodazole, and cells were subsequently collected at four time points (1, 2, 3, and 4 h after release) followed by mixing four populations. Cell lysates were prepared following a hypotonic method described previously (Wan *et al.*, 2001). APC was purified using an antibody against Cdc27. APC were subsequently sub-

jected to a ubiquitination cocktail (50 μ g/ml Ubc5, 1.25 mg/ml ubiquitin, 200 μ g/ml recombinant E1, 0.1 mg/ml cycloheximide, and 2 μ M ubiquitin aldehyde) with 1/10 the volume of ³⁵S-labeled *in vitro*-translated cyclin B. Ubiquitination was reflected in the formation of high-molecular polyubiquitin conjugates. Smeared ubiquitin conjugates were measured by densitometer.

H1 Kinase Assay

Cells were lysed with lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol [DTT], 1 mM EDTA, 0.2% Nonidet P-40, and 5 mM NaF, supplemented with protease inhibitor cocktails). Lysates were incubated with 2 μ g of anti-cyclin B1 antibody (Santa Cruz Biotechnology) overnight at 4°C. Thereafter, protein A/G beads were added to the lysate and incubated for additional 2 h. Immunoprecipitation (IP) complex was washed three times with lysis buffer and once with HBS (10 mM HEPES, pH 7.4, and 150 mM NaCl) before adding the kinase buffer (5 μ g of histone H1 in 20 μ l of HBS containing 15 mM MgCl₂, 50 μ M ATP, 1 mM DTT, and 10 μ Ci of [γ -³²P]ATP) for 10 and 30 min at 30°C. Sample buffer was added to stop the reaction and 20 μ l of each sample was analyzed in 4–12% SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography (Stemmann *et al.*, 2001).

³²P-Labeling *In Vivo*

Cells were incubated in phosphate-free DMEM (Invitrogen) containing 10% dialyzed FCS (Invitrogen). Thereafter, cells were exposed for 1 h in [³²P]orthophosphate (PerkinElmer Life and Analytical Sciences, Boston, MA) at a final activity of 0.4 mCi/ml in phosphate-free DMEM. Then, cells were harvested in IP lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, 2 mM MgCl₂, 1 μ M okadaic acid, and 1 μ M microcystin LR, supplemented with protease inhibitor cocktail). Cell lysates were incubated with 5 μ g of anti-CDC27 antibody (Santa Cruz Biotechnology) or 2 μ g of anti-separase antibody (Novus Biologicals) followed by incubation with protein A/G beads (Pierce Chemical, Rockford, IL). Immunoprecipitated samples were washed once with lysis buffer and three times with wash buffer (20 mM Tris-HCl, pH 7.7, 0.2% NP-40, 1 mM EDTA, 2 mM MgCl₂, 1 μ M okadaic acid, and 1 μ M microcystin LR). Samples were resolved using SDS-PAGE followed by autoradiography (Bakkenist and Kastan, 2003).

Immunoprecipitation

Cells were lysed with lysis buffer (for Cdh1 and Cdc20: 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM, phenylmethylsulfonyl fluoride, 0.5% NP-40, 0.1% SDS, and protease inhibitor cocktail; and for Cdc27: 50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, 2 mM MgCl₂, 1 μ M okadaic acid, and 1 μ M microcystin LR, supplemented with protease inhibitor cocktail), and lysate was incubated with 5 μ g of anti-CDC27 antibody (Santa Cruz Biotechnology) followed by incubation with protein A/G beads (Pierce Chemical). Immunoprecipitated samples were washed and analyzed by SDS-PAGE. Western blot was then performed using anti-CDC27 (Santa Cruz Biotechnology), anti-Cdh1 (Calbiochem), and anti-CDC20 antibody (Liu *et al.*, 2007).

Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100. Immunofluorescent analysis was performed using anti-tubulin (Calbiochem), anti-phospho-smad3 (Cell Signaling Technology, Danvers, MA), and anti-phospho-histone 3 (Millipore, Billerica, MA). Secondary antibodies were Cy2, fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA), and Texas-Red (Jackson ImmunoResearch). For analysis of mitotic cells, number of phospho-histone H3-positive cells was counted among 1000 cells in different fields at least three times (Tang *et al.*, 2006).

RESULTS

Disruption of Smad3 Alters Mitotic Progression in BMSCs

TGF- β 1, a dual-effect growth factor, can act as a potent cytostatic factor for tumor suppression or as an enhancer that promotes tumor malignancy depending on cell type and tumor stage (Siegel and Massague, 2003; Roberts *et al.*, 2006). Previous studies have extensively examined the mechanism by which the cell cycle is regulated by TGF- β signaling, in which a number of critical G1 regulators, such as p15, p21, p27, CDK4.6, and CDK2, are regulated by TGF- β in various types of epithelial cells (Datto *et al.*, 1995; Massague and Chen, 2000; Liu, 2006). Recent studies on stromal cells have sketched the basic framework of TGF- β affecting tumor microenvironment, resulting in subsequent metastasis.

Figure 1. Disruption of Smad3 alters mitotic progression in BMSC. (A) Establishment of bone marrow stromal cells from wild-type or Smad3 knockout mouse. (B) Disruption of TGF- β signaling through targeted deletion of Smad3 mouse cells results in accumulation of G2/M analyzed by FACS. Approximately 44% G2/M population was measured in Smad3^{-/-} cells, whereas only ~25% G2/M cells presented in wild-type BMSCs. Rescue of Smad3 by transduction of retrovirus carrying Smad3 restore the altered cell cycle profile. (C) Alteration of G2/M due to loss of Smad3 is mainly attributed to accumulation of mitotic population. Mitotic population was determined by immunocytochemical analysis using antibody against phosphorylated histone H3 (pH3). pH3-positive cells were accounted and plotted for quantification. Results are mean $\pm$ SD of three independent experiments. (D) Immunocytochemical analysis of primary cultured bone marrow stromal cells from wild-type and Smad3^{-/-} mice. Marrow fluid was collected from femoral bone for isolating bone marrow cells. Mitotic cells were determined by immunocytochemical analysis using anti-phospho-histone H3 antibody. Approximately 5% mitotic cells were measured in wild-type primary cultured cells, whereas ~15% mitotic cells were observed in primary bone marrow stromal cells derived from Smad3 knockout mouse.

sis (De Wever and Mareel, 2003; Dong and Blobel, 2006). To explore the cellular function of TGF- β in stromal cells, we have established Smad3-deficient BMSCs and then dissected the effect of TGF- β on cell cycle progression (Figure 1A).

Disruption of TGF- β signaling by targeted deletion of Smad3 resulted in significant accumulation of BMSC at G2/M (Figure 1B), but not for mouse embryonic fibroblast (MEF) cells (data not shown) based on flow cytometric cell cycle analysis. Re-expression of Smad3 at endogenous levels in Smad3^{-/-} cells returned the cellular population to the original cell cycle profile (Figure 1B). Immunocytochemical analysis of phosphorylation of histone H3, a hallmark of mitosis, has further shown that accumulation at G2/M of

Smad3 knockout cells is due to an increased population of mitotic cells (Figure 2C). Smad3^{-/-} cells have 15% of their total number in mitosis as reflected by positive phospho-histone H3, whereas ~5% of wild-type BMSCs stained positively for phospho-histone H3 cells (Figure 1C). To validate the notion that Smad3 modulates mitotic progression, we collected bone marrow from Smad3^{-/-} mouse and established primary bone marrow stromal cells for mitotic analysis using phospho-histone H3 as readout. As indicated in Figure 1D, ~15% of the primary stromal cells were in mitosis for Smad3^{-/-} mouse, whereas ~5% of wild-type stromal cells were mitosis. In summary, these results suggest that Smad3 mediated TGF- β signaling modulates mitotic pro-

Figure 2. Activation of Smad3 mediates TGF- β signaling in mitosis. (A) Basal TGF- β signaling was detected in wild-type bone marrow stromal cells but not in Smad3^{-/-} cells. Activation of TGF- β signaling was neutralized by supplementation of antibody against TGF- β ligand in the cultured medium. (B) Activation of TGF- β signal was detected in mitosis during the cell cycle. Wild-type BMSCs were synchronized using double thymidine and released. Cells were then collected at different times. Activated TGF- β signaling was measured by Western blotting using antibody against phosphorylated Smad3. (C) Localization of activated TGF- β signal in mitotic chromosomes was quantified by immunostaining of activated Smad3 by using antibody against phosphorylated Smad3 (red).

gression in bone marrow stromal cells. Accumulation of the mitotic population due to an incomplete TGF- β signaling pattern could probably have resulted from failure in the regulation of chromatid separation.

Activation of Smad3 Mediates TGF- β Signaling in Mitosis

Recent studies have suggested a potential involvement of the Smad cascade in regulating the cellular mitotic apparatus (Batut *et al.*, 2007). To examine the effect of TGF- β signaling in the regulation of mitosis, we determined the effect of the presence of activated Smad3 on mitosis by biochemical and immunocytochemical analyses. We first measured the TGF- β response in wild-type or Smad3^{-/-} bone marrow stromal cells by using phospho-Smad3 as readout. As shown in Figure 2A, a basal TGF- β response was detected in wild-type cells, suggesting that basal TGF- β 1 is secreted to the cultured medium, which ensures cellular metabolism. Administration of cells with TGF- β 1 at 100 pM significantly increased the activation of TGF- β pathway, whereas no phospho-Smad3 was detected in the Smad3 deletion cells (Figure 2A). Neutralization of TGF- β ligand by excess supplementation of antibody against TGF- β 1 significantly blocked the activation of TGF- β signaling in the wild-type cells (Figure 2A). To test the status of TGF- β signaling during the cell cycle, we synchronized wild-type BMSCs with a double thymidine block. Cells were then released and collected at different time points for immunoblotting and fluorescence-activated cell sorting (FACS) analyses as indicated (Figure 2B). The result shown in Figure 2B of phosphorylation of Smad3 was measured over a broad range during the

cell cycle. Activation of TGF- β as reflected by phospho-Smad3 is detected during the mitosis (Figure 2B).

To validate the result by immunoblotting, we analyzed the localization of phosphorylated Smad3 in mitotic cells by using an immunocytochemical analysis. As shown in Figure 2C, activated Smad3 was detected in anaphase as well as telophase in wild-type cells, whereas no activated Smad3 was found in metaphase chromosomes in Smad3^{-/-} cells. This result further suggests the role of TGF- β involved in mitotic regulation.

The APC Pathway Is Affected by Deletion of Smad3

To obtain insight into the mitotic apparatus, which mediates the TGF- β response, we systematically analyzed the critical molecules that are involved in mitotic progression (Barr *et al.*, 2004). We conducted an analysis of the protein expression profile of a cluster of mitotic regulators as indicated (Figure 3, A and B). The relative abundance of candidate proteins was measured using immunoblotting, comparing expression in wild-type and Smad3^{-/-} cells. As shown in Figure 3, A and B, the expression of several proteins (phosphorylation and protein levels) exhibited dramatic differences between wild-type and Smad3^{-/-} cells. Among the tested proteins, phosphorylation of CDC27 (reflected by the protein shift) in wild-type cells was significantly stronger than that of CDC27 in Smad3^{-/-} cells. In contrast, phosphorylation of separase (represented by the protein shift) in Smad3^{-/-} cells is much stronger than that of separase in wild-type cells (Figure 3, A and B). Meanwhile, we also noticed the elevated expression of cyclin B in Smad3^{-/-} cells but not in the wild-type cells. Moreover, a smaller

Figure 3. Protein profile of components governing mitotic progression between wild-type and Smad3^{-/-} BMSC cells. (A) Expression and phosphorylation status of several mitotic proteins, including CDC27, cyclin B, and separase, were significantly different between wild-type and Smad3^{-/-} cells. (B) Quantification of protein expression and phosphorylation status for several mitotic proteins in Smad3^{-/-} cells relative to proteins in wild-type cells.

amount of separase cleavage products (C terminus) was measured in Smad3^{-/-} cells than wild-type cells. Given the key function of the APC in governing chromatid separation pathway (Stemmann *et al.*, 2001; Holland and Taylor, 2006), the protein expression profiling suggests that the APC-separation pathway may be the site in the molecular cascade that mediates the TGF- β effect on mitosis.

Modulation of Mitosis by TGF- β Is via Regulating Phosphorylation of CDC27

To confirm the result that TGF- β modulates CDC27 via phosphorylation, we measured the phosphorylation of CDC27 in the presence or absence of Smad3 by using IP immunoblotting and incorporation of phosphate into CDC27 by *in vivo* labeling [³²P]orthophosphate (Bakkenist and Kastan, 2003). As shown in Figure 4A, intense phosphorylation of CDC27 (protein band is shifted and smeared) was detected in wild-type cells, whereas weaker CDC27 phosphorylation was observed in Smad3^{-/-} cells. Consistent with IP immunoblotting experiment, incorporation of radiolabeled phosphate in wild-type cells is about fourfold stronger than that in Smad3^{-/-} cells (Figure 4, B and D). Rescue of Smad3 restored CDC27 phosphorylation as indicated in Figure 4, C and D. This result further suggests that the TGF- β effect in mitosis is to regulate CDC27 through phosphorylation.

Mitotic Defect Due to Loss of Smad3 Results in Elevation of CDK1 via Accumulation of Cyclin B

The measurement of protein expression profile demonstrated that cyclin B significantly accumulated in Smad3 deficient cells (Figure 3, A and B). To test the effect of TGF- β -mediated regulation of cyclin B on mitotic progression, we measured the kinetics of cyclin B after release from nocodazole arrested wild-type and Smad3^{-/-} cells. We further correlated the TGF- β -affected cyclin B with the progression of chromatid separation by using immunocytochemical analysis. As shown in Figure 5A, in wild-type cells, cyclin B protein levels dropped to basal levels after 4-h

release from the nocodazole arrest. In contrast, cyclin B levels were maintained at high levels and only decreased after 8 h after release from mitosis in Smad3^{-/-} cells. Measurement of phospho-histone H3 showed that mitotic progression in Smad3^{-/-} cells was delayed by ~4 h compared with wild-type cells. These results suggest that TGF- β -mediated alteration of cyclin B could affect mitotic progression.

Using FACS coupled with immunocytochemical analyses, we have carefully examined the mitotic progression and chromatid separation in both wild-type and Smad3^{-/-} cells. As shown in Figure 5B, absence of Smad3 resulted in a 6-h delay in mitotic progression. Consistent with the FACS result, the onset of chromatid separation was significantly delayed in Smad3^{-/-} cells relative to wild-type cells.

Given the data showing that TGF- β affects CDC27 phosphorylation and that TGF- β alters cyclin B protein levels, we next asked whether TGF- β -mediated modulation of CDC27 phosphorylation regulated APC activity in the destruction of cyclin B (Choi *et al.*, 1999; Wan *et al.*, 2001). We purified APC from wild-type or Smad3^{-/-} cells during mitosis and analyzed its activity using ³⁵S-labeled and *in vitro*-synthesized cyclin B1 as a putative substrate (Fang *et al.*, 1998). As shown in Figure 5, C and D, targeted deletion of Smad3 in BMSC cells significantly altered APC activity for ubiquitylating cyclin B. Given that cyclin B is an important component of CDK1, we further measured the effect of TGF- β on CDK1 by using a H1 kinase assay (Stemmann *et al.*, 2001). Consistent with the result from the analysis of APC activity, CDK1 activity is significantly higher in Smad3^{-/-} cells than in wild-type cells as reflected by the different levels of cyclin B protein (Figure 5, E and F). Restoration of Smad3 rescued APC activity and therefore reduced CDK1 activity to approximately normal levels (Figure 5, D and E).

Uncontrolled CDK1 Suppresses Chromatid Separation via Phosphorylation of Separase

Comparison of protein profiling has shown a significant difference in separase phosphorylation between wild-type

Figure 4. TGF- β modulates phosphorylation of CDC27 resulting in governing affinity between Cdh1 and APC. (A) Phosphorylation of CDC27 is measured using IP-Western blotting, showing fraction of phosphorylated CDC27 (protein shift) is significantly more in wild-type cells than Smad3 $^{-/-}$ BMSC cells. (B) Measurement of phosphorylation of CDC27 by using *in vivo* labeling of [^{32}P]orthophosphate. Incorporation of radiolabeled phosphate in CDC27 is significantly higher in wild-type than Smad3 $^{-/-}$ BMSC cells. (C) Absent CDC27 phosphorylation in Smad3 $^{-/-}$ cells was restored by re-expressing Smad3 proteins by transduction of retrovirus carried Smad3. (D) Quantification of CDC27 phosphorylation was measured using *in vivo* ^{32}P labeling.

and Smad3 $^{-/-}$ cells (as reflected by shift of the full-length separase band). Although phosphorylated separase is maintained at high levels in Smad3 $^{-/-}$ cells, the production of cleaved C-terminal separase is significantly decreased (Figure 6A). To confirm the effect of TGF- β on the status of separase phosphorylation, we examined the capacity of phosphate incorporation of separase in BMSCs in the presence or absence of Smad3 (Bakkenist and Kastan, 2003). As shown in Figure 6, B and C, disruption of Smad3 lead to a significant increase in phosphate incorporation for separase. Previous studies have suggested that uncontrolled CDK1 activity in mitosis could affect the activation of separase through phosphorylation (Stemmann *et al.*, 2001; Holland and Taylor, 2006). Our results from dissection of the role of Smad3 in mitotic regulation demonstrated that targeted deletion of Smad3 results in down-regulation of CDC27 phosphorylation, which in turn leads to accumulation of cyclin B, thereby elevating CDK1 activity (Figures 3–5). Together, these results suggested a regulatory-axis of TGF- β -modulating chromatid separation through the TGF- β -Smad3-APC-CDK1-separase pathway.

Previous studies have shown the presence of several cell cycle dependent phosphorylation sites on separase (Stemmann *et al.*, 2001). To map the phosphorylation site on separase targeted by CDK1 initiated through TGF- β signaling, we have expressed various mutated separases in Smad3 $^{-/-}$ cells, including Ser1073, Ser1126, Ser1305, Thr1346, Ser1501, Ser1508, Ser1545, and Ser1552, and subsequently measured the incorporation of phosphate into separase. As shown in Figure 6, D–F, replacing Ser by Ala at 1126 in separase significantly abolished phosphorylation of separase. Expression of separase mutant (S1126A) in Smad3 $^{-/-}$ cells significantly restored the Smad3 $^{-/-}$ phenotype (Supplemental Figure 2), suggesting that Ser1126 is critical for mediating TGF- β -induced phosphorylation of separase. The results from these experiments define a novel regulatory

mechanism by which TGF- β signaling modulates mitotic progression through effects on the Cdh1-APC-CDK1-separase cascade (Figure 7).

DISCUSSION

A New Physiological Role for TGF- β in Regulating Mitotic Progression

TGF- β is a multifunctional cytokine that plays complex roles in tumor suppression and tumor promotion depending on cellular context and tumorigenic stage (Siegel and Massague, 2003; Gupta and Massague, 2006; Roberts *et al.*, 2006). In this study, we have identified a new role for TGF- β in modulating mitosis in bone marrow stromal cells. Dysfunction in TGF- β signaling through targeted disruption of Smad3 causes significant delay in mitotic progression. APC, a master mitotic regulator, is targeted by TGF- β signaling for regulation. Regulation of APC by TGF- β results in the control of CDK1 activity. Uncontrolled elevated CDK1 inhibits chromatid separation via phosphorylation of separase. Demonstration of TGF- β -mediated mitotic regulation unravels a previous mystery in which mitotic regulator is down-regulated in response to TGF- β signaling (Choi *et al.*, 1999; Wan *et al.*, 2001) and further explains the presence of activated Smad2/3 detected during mitosis (Batut *et al.*, 2007), which advances our understanding of TGF- β biology.

Prior studies generally focused on epithelial cells have established a paradigm for TGF- β in the regulation of cell cycle. Several critical cell cycle regulators such as p15, p21, p27, CDK4/6, and CDK2 have been shown to respond to TGF- β signaling, suggesting that the G1 phase serves as a window mediating the TGF- β effect during the cell cycle (Datto *et al.*, 1995; Massague and Chen, 2000; Liu, 2006). Recent studies on stromal-epithelial interaction have greatly attracted attention to the role of TGF- β in stromal cells due

Figure 5. Mitotic defect due to absence of Smad3 protein results in elevation of CDK1 via accumulation of cyclin B. (A) Deletion of Smad3 results in accumulation of cyclin B and delay of mitotic progression. Wild-type and Smad3^{-/-} BMSCs were synchronized with nocodazole and released. Protein levels of cyclin B and p33 were measured at different times after release. (B) Mitotic progression and chromatid separation in wild-type and Smad3^{-/-} cells. Mitotic progression was examined by FACS, and progression of chromatids separation was described by immunostaining. (C) Loss of Smad3 leads to decrease of APC activity. APC activity was measured by recapitulating APC activity using purified APC from wild-type or Smad3^{-/-} cells. ³⁵S-labeled in vitro-translated cyclin B was used as a putative substrate. (D) Quantification of cyclin B ubiquitin conjugates catalyzed by APC. (E) Measurement of CDK1 activity by using H1 kinase assay. Elevated CDK1 activity was recovered by transduction of retrovirus carrying Smad3. (F) Quantification of CDK1 activity.

to its importance in orchestrating the microenvironment eliciting onset of tumor malignancy (De Wever and Mareel, 2003; Epperly *et al.*, 2005; Dong and Blobel, 2006). The mitotic phenotype in the absence of Smad3 in hematopoietic stromal cells is not the same as in MEF cells, suggesting that the cellular behavior of BMSCs in response to TGF- β might not be identical to other types of cells. Results from this work suggest that TGF- β is crucial to ensure normal mitotic progression through modulating the APC-chromatid separation pathway. Given that mitosis is a critical step for segregation of two daughter genomes with high fidelity, coordination of multiple pivotal events during mitosis, such as the mitotic checkpoint and chromatid segregation, needs a sophisticated regulatory system

that can ensure precise mitotic progression with a regulatory circuitry on multiple levels. Both failure to control chromatid separation in stromal cells and deregulation of stromal cell cycle due to failure in TGF- β signaling could affect hematopoiesis resulting in tumor metastasis. Dissecting the role of TGF- β in the modulation of mitotic progression in BMSCs has enabled the expansion of the biological function of TGF- β and further enhanced our understanding of hematological malignancy.

TGF- β Modulates Activity of APC via Regulating CDC27 Phosphorylation

APC has been shown to be a critical regulator bridging various environment signals, including genotoxic stress, mitogenic sig-

Figure 6. Disruption of Smad3 leads to elevation of CDK1, which suppresses chromatid separation through phosphorylation of separase. (A) Separase cleavage was impeded in Smad3^{-/-} cells. Increased phosphorylation of separase was detected in Smad3^{-/-} cells, reflected by a shift in full-length separase. Cleavage of separase was impeded in Smad3^{-/-} cells, represented by C-ter separase. (B) Measurement of separase phosphorylation by using *in vivo* labeling of [³²P]orthophosphate. Incorporation of radiolabeled phosphate in separase was significantly increased in Smad3^{-/-} cells than that of wild-type BMSCs. (C) Quantification of measurement for separase phosphorylation. (D) Mapping of phosphorylation site in separase mediated by CDK1. A set of separase mutants tagged with Myc epitope were transfected into Smad3^{-/-} cells followed by incubation with [³²P]orthophosphate. ³²P-labeled myc-separase was immunoprecipitated and resolved by SDS-PAGE followed by autoradiography. Ser1126 was evaluated to be critical for separase phosphorylation. (E) Expression of mutated separase in Smad3^{-/-} cells. (F) Quantification of ³²P incorporation to various separase mutants.

naling, and TGF- β that induce downstream cellular response (Wan *et al.*, 2001; Chabes *et al.*, 2003; Palframan *et al.*, 2006). Regulation of APC could be achieved at several layers, including phosphorylation of APC, CDC20/Cdh1-activators of APC, and Emi1/CDC14-regulators of CDC20 or Cdh1 (Reimann *et al.*, 2001; Vodermaier, 2004). Phosphorylation of CDC27 was thought to be crucial for activation of APC in mitosis (King *et al.*, 1995; Kraft *et al.*, 2003). Results based on the analyses of Cdc27 phosphorylation, cyclin turnover, measurement of APC activity, and immunocytochemical description of mitotic pro-

Figure 7. Diagram of modulation of mitotic progression by TGF- β signaling. TGF- β signaling involves in regulating mitotic progression via the APC-CDK1-separase cascade.

gression in both wild-type and Smad3^{-/-} cells suggested the conclusion that the TGF- β /Smad3-involved mitotic regulation is mediated by regulating APC activity via alteration of Cdc27 phosphorylation (Supplemental Figure 4). Data from the Mad2 depletion experimental suggested that TGF- β /Smad3-involved mitotic regulation is independent from the spindle assembly checkpoint (Supplemental Figure 3). Our data further suggest the presence of an unknown kinase, which mediates the Smad3 transmitted TGF- β signaling for phosphorylation of CDC27. The consequence of TGF- β -induced phosphorylation of CDC27 results in greater affinity between the activator-Cdh1 and CDC27, thereby achieving activation of APC. Given the presence of multiple phosphorylation sites on CDC27, we do not know the TGF- β responsive phosphorylation site in CDC27 at this time. Identifying the kinase mediating the TGF- β -induced CDC27 phosphorylation and determining the phosphorylation site on CDC27 are the aims of further study.

Alteration of CDK1 by TGF- β Results in Regulating the Chromatid Separation

One of major functions of APC in mitosis is to govern cyclin B protein levels through proteolysis (Glutzer *et al.*, 1991).

Cyclin B levels need to be down-regulated in late mitosis enabling lower CDK1 activity, which promotes chromatid separation and exit from mitosis (Stemmann *et al.*, 2001). Previous studies have demonstrated that high CDK1 activity during the mitosis inhibits chromatid separation via phosphorylation of separase (Stemmann *et al.*, 2001; Holland and Taylor, 2006). Among eight mitotic phosphorylation sites in separase, our results have shown that Ser 1126 mediates phosphorylation catalyzed by CDK1, which is consistent with the previous observation (Stemmann *et al.*, 2001; Holland and Taylor, 2006). To confirm the critical role of Ser 1126 in TGF- β regulated chromatid separation, a dominant-negative separase could be engineered and knock-in analysis of Ser 1126 would be desirable to elucidate the mechanism by which separase is regulated through TGF- β -APC-CDK1 cascade. Demonstration that chromatid separation modulated by TGF- β -initiated alteration of CDK1 has revealed a new pathway for the control of chromatid separation.

Recent work suggested the role of APC/Cdc20 in degradation of p21 for G2/M transition (Amador *et al.*, 2007). Given p21 as one of the major downstream of TGF- β signaling, we also asked whether the p21 could be the site for the TGF- β -mediated mitotic regulation. Results based on monitoring p21 kinetics in mitosis and G1 between wild-type and Smad3 knockout cells further confirmed that CDK1 is the target for TGF- β signaling in modulating mitosis (Supplemental Figure 1, A and B).

Possible Role of the Regulation of the Bone Marrow Stromal Cell Cycle by TGF- β in Tumorigenesis

The cellular microenvironment is governed by stromal cells and is crucial for the maintenance of cell function and tissue integrity. An aberrant microenvironmental biology caused by dysfunctional stromal cells could alter the fate of adjacent cells and possibly could promote malignancy (Epperly *et al.*, 2005, 2006; Zhou *et al.*, 2005). In the hematopoietic system, bone marrow stromal cells are integral components of hematopoiesis, and they secrete and respond to cytokines, provide adhesion elements for hematopoietic cells, and create stem cell homing. Regulation of stromal cellular function by TGF- β has been shown to be under the control of the hematopoietic cell lineage. Uncontrolled TGF- β signaling in bone marrow stromal cell alters its cellular properties, including proliferation and cell adhesion, which in turn stimulate secretion of growth factors, thereby enhancing tumor growth and metastasis (Epperly *et al.*, 2005, 2006; Zhou *et al.*, 2005). The present study implicates basal levels for TGF- β signaling as critical to ensure a normal cell cycle for BMSCs, especially mitotic progression. Results from most of the previous studies based on epithelial cells have attracted much attention to the cytostatic effect of TGF- β on regulation G1. Thorough evaluation of TGF- β effects on cell cycle regulation in synchronized cells has not been reported. Whether the TGF- β -mediated mitotic response is solely stromal cell dependent remains unknown. The precise physiological consequence of TGF- β -mediated mitotic regulation in marrow stromal cells is still unknown. Given the critical role of stromal cells in hematopoiesis, the information provided from the present study may be of value in furthering our knowledge of stromal cell biology.

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