FUNCTIONAL CHARACTERIZATION OF TGF-β SIGNALING IN SMAD2- AND SMAD3-DEFICIENT FIBROBLASTS.


1) Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD 20892
2) Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461
3) Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461
4) Cancer and Developmental Biology Laboratory, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD 21702
5) Genetics, Development, and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892

Running title: TGF-β signaling in Smad-deficient cells

Corresponding author:
Ester Piek
Laboratory of Cell Regulation and Carcinogenesis
National Cancer Institute
Building 41, Room C629
41 Library Drive, MSC-5055
Bethesda, Maryland 20814-5055
Fax: 301-496-8395
Phone: 301-496-8347
Email: pieke@mail.nih.gov
SUMMARY

A prominent pathway of TGF-β signaling involves receptor-dependent phosphorylation of Smad2 and Smad3, which then translocate to the nucleus to activate transcription of target genes. To investigate the relative importance of these two Smad proteins in TGF-β1 signal transduction, we have utilized a loss-of-function approach, based on analysis of effects of TGF-β1 on fibroblasts derived from mouse embryos deficient in Smad2 (S2KO) or Smad3 (S3KO). TGF-β1 caused 50% inhibition of cellular proliferation in wild-type fibroblasts as assessed by [³H]thymidine incorporation, while the growth of S2KO or S3KO cells was only weakly inhibited by TGF-β1. Lack of Smad2 or Smad3 expression did not affect TGF-β1-induced fibronectin synthesis, but resulted in markedly suppressed induction of plasminogen activator inhibitor-1 (PAI-1) by TGF-β1. Moreover, TGF-β1-mediated induction of the matrix metalloproteinase MMP-2 was selectively dependent on Smad2, while induction of c-fos, Smad7, and TGF-β1 autoinduction relied on expression of Smad3. Investigation of transcriptional activation of TGF-β-sensitive reporter genes in the different fibroblasts showed that activation of the (SBE)₄-Lux reporter by TGF-β1 was dependent on expression of Smad3, but not on Smad2, whereas activation of the ARE-Lux reporter was strongly suppressed in S2KO fibroblasts but, to the contrary, enhanced in S3KO cells. Our findings indicate specific roles for Smad2 and Smad3 in TGF-β1 signaling.
INTRODUCTION

Transforming growth factor (TGF)-β is the prototypic member of the TGF-β superfamily and mediates a multiplicity of biological effects on different cell types. TGF-β regulates cellular proliferation, induces synthesis of extracellular matrix proteins such as fibronectin and plasminogen activator inhibitor (PAI)-1, modulates the immune response, and plays an important role in embryonic development and cellular differentiation (1).

TGF-β evokes its biological effects by signaling through two different receptor serine/threonine kinases, TGF-β receptor type I (TβR-I) and TβR-II, that form a tetrameric complex following binding of TGF-β to TβR-II. TβR-II activates TβR-I by phosphorylation of serine residues in the GS-box. The anchor protein SARA (Smad anchor for receptor activation) recruits the cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smads), to the TGF-β type I receptor kinase domain, resulting in their phosphorylation on serine residues in the C-terminal SSXS motif. Activated R-Smads heteroligomerize with the common partner Smad4 (Co-Smad) and these complexes are transported into the nucleus where they regulate gene expression. R-Smads and Co-Smads contain two highly conserved domains, the Mad-homology domain 1 (MH1) and the MH2 domain, which are connected by a linker region. While their MH1 domains can interact with the DNA, the MH2 domains are endowed with transcriptional activation properties.

The abbreviations used are: ARE, activin response element; CDKI, cyclin-dependent kinase inhibitors; DFs, dermal fibroblasts; ECM, extracellular matrix; FAST, forkhead activin signal transducer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, knockout; MEF, mouse embryo fibroblast; MH, Mad homology; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; SARA, Smad anchor for receptor activation; SBE, Smad binding element; TβR, TGF-β receptor; TGF-β, transforming growth factor-beta; TPA, tetradecanoyl phorbol acetate; WT, wild-type
Downregulation of TGF-β signaling is effected, in part, by a feedback mechanism involving induction of expression of the inhibitory Smads, Smad6 and Smad7 (anti-Smads), which then prevent R-Smad activation (2;3).

Absence of Smad2 or Smad3 expression resulting from targeted deletion of the respective Smad genes in mice has revealed different developmental roles for Smad2 and Smad3. Homozygous loss-of-function mutations of the Smad2 gene by targeted deletion of the MH1 or MH2 domain resulted in embryonic lethality, due to failure to establish an anterior-posterior axis, gastrulation and mesoderm formation (4;5). These events are controlled by Smad2-dependent signals from the visceral endoderm (6;7). Postgastrulation-rescued Smad2 mutant embryos survived up to embryonic day E10.5 but showed several malformations like cyclopia, cranial abnormalities, and impaired left-right patterning as observed by abnormal heart looping and embryo turning (7).

In contrast, mice harboring homozygous deletions of the Smad3 gene are viable and survive for several months, indicating that Smad3 is dispensable for embryonic development. However, Smad3 knockout mice are smaller than wild-type littermates, and show forelimb malformations (8;9). Mice lacking expression of Smad3 die from chronic inflammation of several organs as a consequence of impaired immune function including defects in mucosal immunity, as revealed by absesses in tissues adjacent to mucosal membranes, and expansion of activated T cell populations. This can be partly attributed to the lack of responsiveness of Smad3-deficient T cells to the growth inhibitory effects of TGF-β, as well as to a defective chemotactic response of Smad3-deficient neutrophils to TGF-β (8;9). Smad3-deficient mice show accelerated wound healing compared to wild-type littermates, which is a consequence of enhanced re-epithelialization by proliferating keratinocytes and reduced wound infiltration as well as TGF-β
production by monocytes (10). Homozygous Smad3 knockout mice generated by Zhu and colleagues (11) die from colon carcinomas between four and six months of age, a phenotype that was not observed in Smad3 null mice derived by Datto et al., (8) or Yang et al., (9).

To investigate the relative importance of Smad2 and Smad3 in TGF-β1 signaling, we have established mouse embryo-derived fibroblasts lacking expression of the Smad2 or Smad3 genes (7;9). In contrast to analysis of the function of Smad2 and Smad3 by over-expression studies, these loss-of-function cell systems provide a more appropriate model to investigate the physiological roles and relative importance of these R-Smads in TGF-β signaling, and provide insight in the consequence of impaired TGF-β R-Smad-function in relation to pathophysiology. Our data show that expression of Smad2 or Smad3 in fibroblasts is important for TGF-β1-mediated growth inhibition as well as for synthesis of PAI-1, while Smad2 and Smad3 contribute uniquely to TGF-β1-induced activation of several luciferase reporter constructs. We further show that certain genes are selectively dependent on only one of these two TGF-β-receptor activated Smads, as, for example, the matrix metalloproteinase MMP-2 which is critically dependent on Smad2 but not Smad3 expression. Collectively, our results indicate non-redundant roles for Smad2 and Smad3 in TGF-β1-mediated signaling and provide insight into the targets of these specific signaling pathways *in vivo.*
TGF-β signaling in Smad-deficient cells

EXPERIMENTAL PROCEDURES

Generation of mouse embryo-derived fibroblasts and primary dermal fibroblasts

Mouse embryo-derived fibroblasts harboring the null allele Smad2\(^{Δex2}\) in the homozygous state were derived as previously described (7). Briefly, ES cells that were wild-type (Smad2\(^{+/+}\), S2WT), heterozygous (Smad2\(^{Δex2/+}\)) or homozygous (Smad2\(^{Δex2/Δex2}\), S2KO) for the Smad2 null allele, were established from F1 heterozygous Smad2\(^{Δex2}\) intercrosses. ES cells were on a C57/BL6x129V mixed genetic background. Tetraploid blastocysts from ROSA 26, B6C3H, or CAF1 females were derived following standard protocols and injected with S2WT or S2KO ES cells. Embryos from 10.5 gestation days were triturated in 0.25% trypsin/1mM EDTA and genotyped as previously described. Cells from 2 embryos with identical genotypes were pooled and cultured, and one S2WT and one S2KO cell line survived and were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, and 50 \(µg/ml\) streptomycin to establish S2WT and S2KO fibroblasts that were cultured over multiple passages to obtain sufficient cells to perform the experiments.

Smad3 knockout mice were generated by targeted deletion of exon 8 in the Smad3 gene by homologous recombination as described previously (9). Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring. Embryos were triturated in 0.25% trypsin/1mM EDTA and genotyped as previously described (9). Cells from several Smad3 wild-type (Smad3\(^{+/+}\); S3WT) and knockout (Smad3\(^{Δex8/Δex8}\); S3KO) littermate embryos (17d) were pooled by genotype to generate fibroblasts, which were used for experiments at passage 3 as primary mouse embryo fibroblasts (MEFs), or were routinely cultured over multiple passages in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 50 \(µg/ml\) streptomycin to
establish fibroblasts. The Smad2 and Smad3 fibroblasts used for the experiments shown were used at passage number 20-35. Three different S3KO and S3WT fibroblast lines were tested in the different experiments, giving similar results, and representative data are shown. The results obtained were independent of the passage number.

Primary dermal fibroblasts (DFs) from S3WT and S3KO newborn pups were established as described by Glick et al., (12), and were used at passage 2.

Mouse NMuMG mammary tumor cells were cultured in DMEM with 10% FBS, 100 units/ml penicillin and 50 µg/ml streptomycin.

Western Blot analysis

Plasmids encoding flag-tagged Smad2 (pflSmad2) and flag-tagged Smad3 (pflSmad3) were kindly provided by Dr. J. Wrana (Mount Sinai, Toronto, Canada) and Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, NY), respectively. The expression construct encoding truncated Smad3 was created as described previously (9). Recombinant adenovirus expressing Smad3 or β-galactosidase were obtained from Dr. K. Miyazono (The Cancer Institute, Tokyo, Japan) and were used at MOI 40 to infect primary S3KO MEFs and S3KO DFs as described by Fujii et al., (13). Flag-tagged Smad2, flag-tagged Smad3 and truncated Smad3 were over-expressed in COS-1 cells and proteins were extracted in RIPA buffer (125 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X-100). S3WT and S3KO fibroblasts, MEFs, DFs, as well as lung tissue from S3WT or S3KO transgenic mice were lysed in RIPA buffer to obtain protein extracts. Protein concentrations were measured using the Bio-Rad protein assay according to the protocol provided by the manufacturer. Protein samples (100 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis followed by wet-transfer to Immobilon-P membranes.
TGF-β signaling in Smad-deficient cells

(Millipore). Nonspecific binding of proteins to the membranes was blocked in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% milk. Western blot analysis was performed using the rabbit polyclonal Smad2 or Smad3 primary antibodies (Zymed), and horseradish peroxidase-conjugated donkey anti-rabbit Ig secondary antibody (Amersham, Life Science). To detect matrix metalloproteinase 2 (MMP-2) in gelatin sepharose-purified conditioned medium from the fibroblasts (described below), a polyclonal rabbit anti-human MMP-2 antibody, kindly provided by Dr. W.G. Stetler-Stevenson, was used (14). Detection was performed by enhanced chemiluminescence (ECL).

Proliferation Assays

Cell proliferation was measured by [3H]thymidine incorporation as described by Danielpour et al. (15). Briefly, WT, S2KO and S3KO fibroblasts (1x10⁴ cells/well) were seeded into 24-well dishes in DMEM/10% FBS. Following attachment (2h), TGF-β1 was added to the culture medium for an additional 24h at varying doses as indicated. The cells were pulsed with 0.5 μCi/well of [3H]thymidine for the last 1h of incubation, and incorporated label was determined as described (15).

Northern blot analysis and cDNA probes

Total RNA was isolated from cells using the RNeasy column purification method (Qiagen, Santa Clara, CA) following the manufacturer’s protocol. For Northern blot analysis, RNA (10 μg) was electrophoresed on 1% agarose gels and transferred to Nytran-N nylon membrane (Schleicher & Schuell). Membranes were hybridized with the relevant [32P]-labeled cDNA probes in Church buffer (16) or QuickHyb solution (Stratagene) according to the manufacturer's
protocol, and analyzed by phosphorimagery or by exposure to Kodak BioMax MS films. Equal RNA loading was assessed by ethidium bromide-staining of 28S and 18S rRNA, or alternatively, membranes were hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S rRNA cDNA probe. Hybridizations were performed using the following cDNA inserts: 1300-bp NheI/XhoI fragment of murine p15INK4B, 420-bp EcoRI fragment of murine p21CIP1/WAF1, 650-bp NdeI/XhoI fragment of mouse p27Kip1, 2200-bp BamHI/HindIII fragment of rat c-fos, 1300-bp EcoRI/XhoI fragment of murine Smad7, and 1000-bp HindIII/XbaI fragment of rat TGF-β1.

**Extracellular matrix protein assays**

TGF-β1-induced fibronectin and PAI-1 synthesis was assayed as described by Wrana et al., (17). MMP-2 was co-purified with fibronectin using gelatin-sepharose beads in the fibronectin assay. MMP-2 zymography was basically performed as described by Kleiner and Stetler-Stevenson (18), using gelatin sepharose affinity-purified proteins from fibroblast-conditioned medium. Briefly, protein samples were separated under non-reducing conditions on an 8% SDS polyacrylamide gel containing 1 µg/ml gelatin. The gel was incubated for 1hr at room temperature in 2.5% Triton X-100 on a rotary shaker, followed by incubation for 18hr at 37°C in enzyme buffer containing 50 mM Tris, pH7.5, 200 mM NaCl, 5mM CaCl2, and 0.02% Brij-35. The gel was washed in H2O, stained with Gelcode Blue Stain Reagent (Pierce, Rockford, IL) according to the manufacturer’s protocol, and dried on 3MM paper.

**Transcriptional reporter assays**

3TP-Lux reporter construct, ARE luciferase reporter construct as well as forkhead activin signal transducer (FAST)-1 construct, and (SBE)4-luciferase reporter construct were provided by
TGF-β signaling in Smad-deficient cells

Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, NY), Dr. M. Whitman (Dept. of Cell Biology, Harvard Medical School, Boston) and Dr. P. ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden), respectively. For transcriptional reporter assays, fibroblasts were seeded at a density of $10^5$ cells per 6-well dish. The next day, cells were transfected with the different luciferase reporter constructs using FuGene6 transfection reagent (Boehringer Mannheim, Germany) according to the manufacturer’s protocol. For Smad reconstitution experiments, pf1Smad2 or pf1Smad3 were co-expressed with the respective luciferase reporter constructs. Cells were transfected for 30h followed by stimulation with indicated concentrations of TGF-β1 for 18h. In all transfections the expression plasmid pSV-β-galactosidase served as internal control to correct for transfection efficiency.
RESULTS

Analysis of Smad expression in S2KO and S3KO fibroblasts

Fibroblasts deficient in Smad2 or Smad3 gene expression were derived from mice in which the respective Smad alleles are disrupted by homologous recombination resulting in targeted deletion of exon 2 in Smad2 (7), and exon 8 in Smad3 (9), respectively. Western blot analyses of cellular lysates derived from S2KO fibroblasts and S2KO ES cells using four different antibodies against various peptide sequences in the N-terminal and C-terminal domains of Smad2 demonstrated that the Smad2 deletion in exon 2 resulted in a null allele (7;19). Western blot analysis of lysates from S3WT and S3KO fibroblasts, primary MEFs, primary DFs, or lung tissue, showed that S3WT cells and S3WT lung tissue express full length Smad3, whereas neither full length nor truncated Smad3 protein could be detected in S3KO cells or S3KO lung tissue using a specific antibody that was raised against the Smad3 middle linker region (Fig. 1A). The antibody did detect truncated Smad3 that was over-expressed by transient transfection in COS-1 cells.

Although we were unable to detect expression of truncated Smad3 protein in tissues or fibroblasts derived from S3KO mice, we assessed whether expression of a putative truncated Smad3 protein could interfere with Smad2 signaling by using a Smad2-dependent transcriptional reporter assay (20;21). Using NMuMG murine mammary gland epithelial cells, we observed that TGF-β1 efficiently induced the activin response element (ARE)-luciferase reporter in the presence of co-expressed forkhead activin signal transducer FAST-1, and that this induction was further enhanced following co-expression of Smad2 (Fig. 1B). Whereas over-expression of full length Smad3 abrogated the TGF-β1-dependent activation of the ARE reporter mediated by Smad2/FAST-1, as previously reported (22;23), expression of truncated Smad3 enhanced ARE
reporter activation, presumably by its ability to act as a dominant negative inhibitor of endogenous Smad3 (9). These data suggest that even if it were expressed, this truncated version of Smad3 would not interfere with Smad2-mediated TGF-β1 signal transduction (Fig. 1B).

Loss of basal proliferation and TGF-β1 growth inhibitory response in Smad2- and Smad3-deficient fibroblasts

Although TGF-β can stimulate proliferation in several fibroblast cell lines, it is known to inhibit the growth of primary MEFs (8;24). We examined the incorporation of [3H]thymidine to examine the roles of Smad2 and Smad3 in control of basal rates of proliferation and in transducing growth control signals by TGF-β1 in the different fibroblasts.

Basal rates of [3H]thymidine incorporation were 2-fold higher in S2WT compared to S2KO fibroblasts, and 8-fold higher in S3WT compared to S3KO cells (Fig. 2). Inspection of cell cultures during the experiments did not reveal significant differences in cell attachment, cell death, or in cell densities before and after labeling. These results indicate that lack of either Smad2, or in particular Smad3 is associated with decreased cellular proliferation rates in regular growth medium.

In low-density cultures (10,000 cells seeded per 24-well dish) of S2WT or S3WT fibroblasts, TGF-β1 treatment for 24h reduced [3H]thymidine incorporation by 46% (P=0.001) and 54% (P=0.01) when compared with untreated cells, respectively (Fig. 2). In contrast, TGF-β1 had only a modest effect on [3H]thymidine incorporation in S2KO (12% reduction) and S3KO fibroblasts (16% reduction) compared with untreated cells, respectively. At higher plating density (15,000 cells per 24-well dish), [3H]thymidine incorporation was inhibited in response to TGF-β1 by 36% in S3WT and 9% in S3KO fibroblasts, and stimulated by 13% in S2KO
fibroblasts compared to untreated cells, respectively (data not shown). Our observations demonstrate that lack of either Smad2 or Smad3 markedly reduces the sensitivity of fibroblasts to growth inhibition by TGF-β1. However, it should be noted that the relative absence of a growth inhibitory response in these cells is associated with already substantially reduced basal growth rates when compared to the relevant WT fibroblasts.

Defects in p15 and p21 regulation in Smad-deficient fibroblasts

Regulation of the cyclin-dependent kinase inhibitors (CDKIs) p15\textsuperscript{INK4B}, p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{Kip1} has been shown to mediate cell cycle control by TGF-β, depending on cell type and context (25). Since both S2KO and S3KO fibroblasts showed reduced basal growth rates and had lost growth inhibitory responses to TGF-β1 (see Fig. 2), we examined the effect of lack of Smad2 or Smad3 on CDKI expression at baseline and in response to TGF-β1.

Northern blot analysis showed that TGF-β1 treatment of S2WT fibroblasts resulted in early repression (3.9-fold) of p15 mRNA at 1h, but a 2.1-fold induction at 10h. A similar profile was observed in S3WT fibroblasts following TGF-β1 treatment, with a 1.8-fold induction at 24h (Fig. 3A). Baseline p15 mRNA levels were reduced by 33% in S2KO and by 35% in S3KO fibroblasts compared with their WT controls, respectively, and treatment of fibroblasts with TGF-β1 stimulated p15 expression in the absence of Smad2, but not in the absence of Smad3. Importantly, mRNA levels in both S2KO and S3KO fibroblasts in response to TGF-β1 remained significantly reduced when compared to WT cells at all time points examined, in particular at 10h and 24h.

In striking contrast with p15 expression, baseline mRNA levels of p21 were dramatically increased in S2KO (22-fold) and S3KO fibroblasts (9.3-fold), compared with WT cells (Fig. 3B).
TGF-β signaling in Smad-deficient cells

In both S2WT and S3WT fibroblasts, TGF-β1 gradually induced mRNA levels of p21, peaking at 5.4-fold and 5.1-fold induction at 10h when compared to baseline. In contrast, TGF-β1 had little effect on the already elevated levels of expression of p21 mRNA in S2KO cells. In S3KO fibroblasts, TGF-β1 induced a 2.7-fold increase in expression at 1h, but only a modest increase of 1.4-fold at 10h compared to baseline. However, absolute p21 mRNA levels were considerably higher at all time points in both KO cell types compared with the respective WT fibroblasts.

Neither baseline mRNA levels of p27, nor the response of p27 mRNA expression to TGF-β1 treatment were significantly altered in S2KO or S3KO fibroblasts compared with their respective WT controls (Fig. 3C). In all genotypes, TGF-β1 treatment reduced p27 mRNA levels. In addition, expression of c-myc and p53 mRNA was not affected by differences in genotypes or TGF-β1 treatment (data not shown).

Taken together, these observations indicate that Smad2 and Smad3 are each necessary in mediating effects of TGF-β1 on cell proliferation in fibroblasts established from mouse embryos and that absence of either Smad2 or Smad3 severely disturbs both the basal and the TGF-β-dependent regulation of \( p15^{INK4B} \) and \( p21^{CIP1/WAF1} \) expression.

Induction of the immediate-early genes c-fos and Smad7, as well as autoinduction of TGF-β1 is dependent on Smad3

In order to ascertain whether Smad2 or Smad3 are required for the induction of early response genes by TGF-β1, we examined the expression profiles of c-fos and Smad7. TGF-β1 strongly induced steady-state mRNA levels of c-fos at 1h in S2WT and S2KO fibroblasts, but had no significant effect on c-fos expression in S3KO fibroblasts (Fig. 4A). Lack of Smad2 had no significant effect on the induction of Smad7 by TGF-β1 compared with S2WT fibroblasts (Fig.
TGF-β signaling in Smad-deficient cells

4B). TGF-β1-mediated increase of Smad7 mRNA was significantly reduced in S3KO fibroblasts compared with S3WT at 1h (2.7-fold vs. 4.9-fold, respectively) and at 4h (1.1-fold vs. 2.9-fold, respectively). The importance of Smad3 in induction of Smad7 mRNA expression by TGF-β1 was further confirmed in primary MEFs and primary DFs. Thus, adenoviral-mediated reintroduction of Smad3 expression in the S3KO cells (Fig. 1A) restored induction of Smad7 mRNA expression by TGF-β1 (Fig. 4C), whereas infection with recombinant adenovirus expressing β-galactosidase had no effect (data not shown). These observations demonstrate that Smad3 plays an important role in the induction of c-fos and Smad7 expression by TGF-β1.

It is well known that TGF-β1 can induce its own gene expression, partly through the Ras/MAPK signaling pathway which impinges on the transcriptional activation complex AP-1 (26;27). Analysis of TGF-β1 autoinduction in the fibroblasts revealed that both basal and autoinduced expression of TGF-β1 was strongly suppressed in S3KO cells, in contrast to S2KO cells (Fig. 4D). This observation was further confirmed in primary S3WT and S3KO MEFs and DFs. Thus, adenoviral reintroduction of Smad3, but not β-galactosidase (data not shown), in the primary S3KO MEFs and DFs restored autoinduction of TGF-β1 to levels observed in S3WT cells (Fig. 4E). Due to early embryonic lethality of the S2KO mice and the technical complications to derive embryonic fibroblasts, we were unable to perform similar experiments for primary S2WT and S2KO MEFs and DFs. In conclusion, these data show that Smad3 plays an important role in autoregulation of TGF-β1 expression in both primary as well as established fibroblast cell cultures.

_TGF-β1-induced expression of extracellular matrix proteins is partially dependent on Smad2 and Smad3 expression_
TGF-β signaling in Smad-deficient cells

TGF-β is known to induce synthesis of several extracellular matrix proteins, including fibronectin and PAI-1, in many different cell types, and these have recently been shown to be Smad-independent and Smad-dependent, respectively (8;28). As shown in Figure 5A, induction of fibronectin synthesis by TGF-β1 was similar in WT fibroblasts as in S2KO or S3KO fibroblasts, consistent with the observation that Smad4 is dispensable for TGF-β1-induced fibronectin synthesis and that expression is instead dependent on expression and activation of c-Jun N-terminal kinase (28). However, it can not be ruled out that Smad2 and Smad3 can substitute for each other in induction of fibronectin synthesis.

As shown in Figure 5A, a TGF-β1-induced 72 kDa protein co-purified with fibronectin in these assays. Based on its molecular weight, the fact that it was secreted into the conditioned medium, its affinity for gelatin, and its inducibility by TGF-β1, we hypothesized that this protein could possibly represent collagenase IV/72-kDa gelatinase/matrix metalloproteinase 2 (MMP-2), which has been reported to be induced by TGF-β in fibroblasts (29). Indeed, we could detect the 72 kDa protein using an MMP-2 antibody, while its gelatinase activity was shown by zymography (Fig. 5B). Interestingly, TGF-β1-enhanced expression of MMP-2 was Smad2-dependent, and did not require Smad3. In contrast, tissue inhibitor of metalloproteinase (TIMP)-2, which associates with MMP-2 and can thereby also be isolated using gelatin sepharose beads, is known to be induced by TGF-β in several cell types, and its induction by TGF-β1 appeared to be dependent on both Smad2 and Smad3 (data not shown).

TGF-β1-mediated PAI-1 protein synthesis was also reduced in both S2KO and S3KO fibroblasts, compared to WT cells (Fig. 5C). The importance of Smad3 in induction of PAI-1 protein expression by TGF-β1 was further validated in primary MEFs and DFs, where adenoviral reintroduction of Smad3 expression in primary S3KO fibroblasts resulted in full recovery of
PAI-1 induction by TGF-β1. As mentioned above, the early embryonic lethality of S2KO mice precluded us from performing similar experiments in primary S2KO fibroblasts. Thus, for the most part, Smad2 and Smad3 have overlapping roles in TGF-β1-mediated induction of extracellular matrix proteins.

**Role of Smad2 and Smad3 in activation of TGF-β-induced transcriptional reporters**

Receptor-activated Smad proteins including Smad2 and Smad3 function as transcriptional regulators in the nucleus. Whereas over-expression studies have shown that both Smad2 and Smad3 can activate transcription of a variety of TGF-β-dependent luciferase gene reporters, only Smad3 can directly interact with Smad binding elements found in the promoters of many TGF-β-responsive genes (30-33). To investigate the effect of loss of each of these two TGF-β-activated R-Smads on the transcriptional regulation of TGF-β-sensitive reporter genes, we performed transfection studies in the fibroblasts. As shown in Figure 6A, lack of Smad2 expression only slightly reduced activation of the 3TP-Lux reporter (6.2 vs. 8.7-fold induction in S2KO vs. S2WT fibroblasts, respectively) which is driven by part of the PAI-1 promoter and three tetradecanoyl phorbol acetate (TPA)-responsive elements (17). In contrast, absence of Smad3 expression more strongly impaired 3TP-Lux reporter activation by TGF-β1 (2.2 vs 5.1-fold induction in S3KO vs. S3WT fibroblasts; Fig. 6B). Reconstitution of Smad2 or Smad3 expression in the respective S2KO or S3KO fibroblast cell lines restored TGF-β1-mediated activation of the 3TP-Lux reporter to levels achieved in WT cells (Figs. 6A,B).

TGF-β1-induced activation of the (SBE),-luciferase reporter, driven by four repeats of the CAGACA sequence identified as Smad binding element in the *JunB* promoter (34), was dependent on expression of Smad3, but not on Smad2 (Fig. 6C,D), consistent with the inability
of Smad2 to bind DNA. Reconstitution of Smad3 by transient over-expression resulted in efficient activation of the (SBE)$_4$-luciferase reporter in S3KO fibroblasts. Similar results were obtained in primary MEFs and primary DFs, where adenoviral-based reconstitution of Smad3 expression in S3KO fibroblasts also restored the ability of TGF-β1 to induce (SBE)$_4$ luciferase reporter activation (data not shown).

As previously reported (22) and described above (see Fig. 1B), Smad2 and Smad3 differentially affect TGF-β-induced activation of the ARE-luciferase reporter when over-expressed. To address the effect of loss of each of these Smad proteins, S2KO and S3KO fibroblasts were transfected with the ARE-luciferase reporter and FAST-1. Although the fold-induction of ARE-luciferase reporter activity by TGF-β1 was comparable in WT and S2KO fibroblasts, we repeatedly observed that the overall ARE-luciferase reporter levels were suppressed in S2KO fibroblasts (Fig. 4E). On the other hand, absence of Smad3 expression enhanced both absolute levels of activation as well as the fold-induction of the ARE-luciferase reporter by TGF-β1 (Fig. 4F), supporting a suppressive role for endogenous Smad3 in activation of the ARE-luciferase reporter (22). Together, these reporter activation studies clearly indicate differential roles of Smad2 and Smad3 in the induction of specific TGF-β1 target genes.
DISCUSSION

We have investigated TGF-β signaling in established mouse embryo-derived fibroblasts deficient in expression of Smad2 or Smad3, to assess the effect of loss of each of these key signaling intermediates on induction of target gene expression by TGF-β1. We have identified target genes with Smad2- or Smad3-independent patterns of induction, those which are affected by the loss of either R-Smad, and genes which are selectively dependent on one or the other of these two R-Smad proteins. As examples, we have shown that TGF-β1-induced fibronectin synthesis occurs in the absence of Smad2 or Smad3 expression, while both Smads have roles in induction of PAI-1 protein and in the more complex endpoint of TGF-β1-induced growth inhibition with associated regulation of cyclin/cdk inhibitors p15^{INK4B} and p21^{CIP1/WAF1}. We also show for the first time that TGF-β1-mediated induction of c-fos expression requires Smad3, and that induction of matrix metalloproteinase MMP-2 is selectively dependent on Smad2. Moreover, similar to that shown for Smad3 null macrophages and keratinocytes (10), we show that autoinduction of TGF-β1 in fibroblasts is strongly suppressed in the absence of Smad3. To test that results shown previously in over-expression systems are truly dependent on Smad2 and Smad3, we have also assessed the activation of several TGF-β-sensitive reporter genes in these Smad-deficient fibroblasts. Together, these experiments demonstrate that Smad2 and Smad3 have both overlapping and distinct roles in TGF-β1 signaling dependent on target gene and cellular context.

Because of the early embryonic lethal phenotype of the Smad2 knockout mice (4;5), and the technical difficulties involved in derivation of S2KO embryonic fibroblasts, we were forced to do most of our comparisons between the role of Smad2 and Smad3 in TGF-β1 signaling using spontaneously immortalized fibroblasts that were cultured over multiple passages. To underscore
the validity of our studies, we show that expression of Smad3 is also important for TGF-β1-mediated induction of Smad7, TGF-β1 and PAI-1 in primary MEFs and primary DFs. We also show that induction of expression of these genes by TGF-β1 can be restored following stable reintroduction of Smad3 in these primary Smad3-deficient fibroblasts. In contrast, whereas adenoviral- or retroviral-mediated restoration of Smad2 or Smad3 expression in fibroblasts could restore TGF-β1 responsive reporter gene induction dependent directly on Smads, this strategy was not sufficient to restore induction by TGF-β1 of endogenous gene responses shown to be dependent on Smad2- or Smad3 (data not shown). Similar observations of the inability to rescue responses by stable introduction of Smads or other signaling molecules into established cell systems have been reported (35-36). Our data suggest that restoration of Smad expression is not sufficient to fully revert the established Smad knockout fibroblasts to their WT counterparts, possibly because loss of Smad expression in combination with multiple genetic alterations, inherently associated with immortalization, irreversibly alters expression of additional genes important to mediate signaling to more complex endogenous targets of TGF-β.

In contrast to previous reports that address the role of different Smads by over-expression in in vitro systems, we show that fibroblasts derived from mouse embryos lacking expression of Smad2 or Smad3 provide a suitable ‘loss of function’ model system to investigate the different effects of these two R-Smads in TGF-β signaling, as is important for the understanding of their distinct roles in vivo. For example, the different roles of Smad2 and Smad3 are evident in studies of embryogenesis where targeted deletion of Smad2 or Smad3 results in either early embryonic lethality or viable offspring, respectively (4-9;11). In wound healing, decreased levels of Smad2 or Smad3 have dramatically different effects (10). Differences are also apparent in carcinogenesis where Smad2 has been classified as a tumor suppressor, based on its mutation
TGF-β signaling in Smad-deficient cells

frequency in several types of cancer (37;38), but where evidence for a similar role of Smad3 is lacking (39;40). Moreover, since autoinduction of TGF-β1, previously shown to involve Ras/MAPK/AP-1 signaling (26;27), is also dependent on Smad3 (Fig. 4D,E) (10), retention of Smad3 might be selected for in tumor cells, as TGF-β1 secreted by tumor cells can promote tumorigenesis, by inducing metastasis, invasion, and angiogenesis (reviewed in 41). It should be noted, however, that R-Smad activity can be blocked by certain oncogenes including Evi-1 which, in certain cells, could have effects similar to its loss by genetic defects (42).

A number of physical differences between Smad2 and Smad3 have been described that might underlie or contribute to their observed functional differences or, in other cases, to their interchangeability. While the MH1 domain of Smad3 can interact directly with SBE sequences (CAGAGTCT) in the DNA, Smad2 contains an extra exon which encodes 30 amino acids that are absent in the MH1 domain of Smad3 and which prevents its binding to DNA (31;32). Consistent with this, we observed that TGF-β1-induced activation of the (SBE)₄-luciferase reporter, which consists of four concatemerized SBEs derived from the mouse JunB promoter (34), occurred as efficiently in S2KO as in WT fibroblasts, while in S3KO cells, TGF-β1-induced (SBE)₄ luciferase reporter activation was impaired. This is in agreement with previous observations that Smad2, in contrast to Smad3, could not be detected in Smad-complexes bound to a JunB probe, and that over-expression of Smad2 contributed only weakly to TGF-β1-induced activation of the (SBE)₄-luciferase reporter, while over-expression of Smad3 potently enhanced reporter-induction even in the absence of TGF-β (34). In a similar manner, Smad3 is involved in activation of the Smad7 gene promoter, while Smad2 does not have a functional role in its induction by TGF-β (19;43). Interestingly, mechanisms exist to alter the DNA binding patterns of Smads 2 and 3. An alternative splice variant of Smad2 that lacks exon 3 does bind to DNA.
TGF-β signaling in Smad-deficient cells

(32;44), might possibly compensate for loss of Smad3 in mediating activation of certain TGF-β-induced responses.

Differential in vivo activities of Smad2 and Smad3 on the same target element are also supported by our studies, suggesting that Smad2 and Smad3 might have distinct affinities for different transcription factors, and thereby contribute differently to TGF-β signaling. Thus, over-expression of Smad3 inhibits activation of the goosecoid or Mix2 (ARE) TGF-β-target gene promoters that are dependent on forkhead activin signal transducer (FAST), Smad2 and Smad4 (20;21). This inhibition has been proposed to result either from competition between Smad3 and Smad4 for binding to FAST (23) or by competitive affinities of Smad3 and Smad4 for SBE elements in the gene promoters (22). Our loss-of-function studies further support the dependence of this promoter on Smad2 as well as its negative regulation by endogenous Smad3. Thus, we show compromised activation of the ARE luciferase reporter in Smad2-deficient fibroblasts, contrasted with enhanced activation of this reporter in Smad3-deficient cells, as well as in NMuMG cells in which a truncated, dominant-negative form of Smad3 is over-expressed, likely interfering with the function of the endogenous protein (9).

In contrast to direct activation of immediate early genes and TGF-β-sensitive luciferase reporter genes that are likely controlled by low signaling thresholds, regulation of cell growth requires continuous signaling to modulate the tightly balanced cell cycle apparatus that integrates multiple signals at several complex endpoints. It is therefore more difficult to identify the genes that are directly involved in abrogation of TGF-β-induced inhibition of cellular proliferation. Similar to our findings in Smad2- or Smad3-deficient fibroblasts, it has been reported that the growth inhibitory effects of TGF-β are lost in Smad3-deficient MEFs and astrocytes (8;45). Whereas Datto and colleagues (8) did not observe changes in the induction of p15INK4B or
p21\textsuperscript{CIP1/WAF1} in Smad3-deficient MEFs, basal expression levels of p15 and p21 were dramatically decreased and increased, respectively, in our S2KO and S3KO fibroblasts compared with WT controls. In agreement with this, suppressed p15 levels and reduced activation of p15 by TGF-β has been observed in Smad3-deficient astrocytes (45). However, the correlation between dysregulated p15 and p21 levels and the observed growth behavior of our KO fibroblasts is unclear at present.

The results presented here demonstrating that activation of genes by TGF-β1 is often dependent on one of the two TGF-β R-Smads, Smad2 and Smad3, suggest that the observed differences in KO phenotypes of Smad2- versus Smad3-deficient mice are not merely a consequence of differential spatially and temporally controlled patterns of gene expression of these two R-Smads during development, but rather that they have unique, non-overlapping roles in control of target gene expression, which allows for more versatility in cross-talk with other signal transduction pathways. Detailed analysis of TGF-β target gene expression in Smad2 versus Smad3 KO cell systems now has the potential to provide insights into their respective roles in regulation of genes that are of critical importance for both normal physiology and development as well as in disease pathogenesis, including carcinogenesis.

ACKNOWLEDGEMENTS

We would like to thank Dr. J. Wrana for Flag-tagged Smad2 (pf1Smad2), Dr. J. Massagué for Flag-tagged Smad3 (pf1Smad3) and 3TP-Lux reporter construct, Dr. P. ten Dijke for (SBE)₃-luciferase reporter construct, Dr. M. Whitman for FAST-1 and ARE-luciferase reporter constructs, Dr. W. G. Stetler-Stevenson for MMP-2 antibody and valuable advice, Dr. K. Miyazono for recombinant Smad3 and β-galactosidase adenovirus, and Dr. K. C. Flanders and
TGF-β signaling in Smad-deficient cells

Dr. M. Fujii for preparation of the dermal fibroblasts. This work was supported by National Institutes of Health Grant DK-56077-01 (to E.P.B.).
REFERENCES


TGF-β signaling in Smad-deficient cells


TGF-β signaling in Smad-deficient cells


TGF-β signaling in Smad-deficient cells

FIGURE LEGENDS

Figure 1: Targeted deletion of Smad2 or Smad3 results in null alleles. A) Western blot detection of Smad3 in protein extracts from S3WT and S3KO mouse embryo-derived fibroblasts, primary mouse embryo fibroblasts (MEFs), primary dermal fibroblasts (DFs), as well as lung tissue. Primary S3KO MEFs and S3KO DFs were infected with adenoviral Smad3 or adenoviral β-galactosidase (β-gal) at multiplicity of infection (MOI) 40. Smad2, Smad3 and truncated Smad3 were over-expressed in COS-1 cells and protein extracts were used as controls. The Smad3-specific antibody was raised against the linker region of Smad3. B) Effect of truncated Smad3 on Smad2/FAST-1-mediated ARE reporter induction. NMuMG cells were transfected with the ARE-luciferase reporter and FAST-1 in combination with the indicated Smad proteins (white bars, control; black bars, 80 pM TGF-β1). Representative results are shown as the average of triplicate observations, corrected for transfection efficiency as measured by β-galactosidase activity.

Figure 2: Characteristics of cell growth responses in Smad2- and Smad3-deficient fibroblasts. [³H]Thymidine incorporation was measured in counts per minute (cpm) in different fibroblast genotypes in the absence (white bars) or presence of 100 pM TGF-β1 for 24h (black bars). Representative experiments performed in triplicate are shown. Error bars indicate standard deviation from the mean in triplicate experiments.

Figure 3: Expression of inhibitors of cyclin-dependent kinases (CDKIs) in response to TGF-β1 in fibroblasts deficient in Smad2 or Smad3. Northern blot analysis of A) p15INK4B, B) p21Waf1/Cip1 and C) p27Kip1 steady-state mRNA expression in total RNA lysates from S2WT, S2KO, S3WT
and S3KO fibroblasts exposed to 100 pM TGF-β1 for the indicated time periods. Membranes were probed for GAPDH mRNA levels to normalize for RNA loading. Bar graphs show relative steady-state expression levels of CDKIs measured by densitometry after normalization for GAPDH (white bars, S2WT; light gray bars, S2KO; dark gray bars, S3WT; black bars, S3KO). Representative exposures from at least two independent repeats per experiment are shown.

Figure 4: Expression of immediate-early response genes c-fos and Smad7, and TGF-β1 autoinduction. Northern blot analysis of A) c-fos and B) Smad7 steady-state mRNA expression in total RNA lysates from S2WT, S2KO, S3WT and S3KO fibroblasts exposed to TGF-β1 for the indicated time periods. Membranes were probed for GAPDH mRNA levels, and quantitative analysis of normalized mRNA expression is shown (white bars, S2WT; light gray bars, S2KO; dark gray bars, S3WT; black bars, S3KO). Representative exposures from at least two independent repeats per experiment are shown. C) Smad7 steady-state mRNA expression in total RNA lysates from primary S3WT and S3KO MEFs and DFs, exposed to TGF-β1 for the indicated time periods. Smad3 expression was reintroduced in S3KO cells by adenoviral infection at MOI 40. Ethidium bromide-stained 18S rRNA bands are shown as RNA loading controls. D) TGF-β1 steady-state mRNA expression in total RNA lysates from S2WT, S2KO, S3WT and S3KO fibroblasts exposed to TGF-β1 for the indicated time periods. Membranes were probed for 18S rRNA to control for RNA loading. E) TGF-β1 steady-state mRNA expression in total RNA lysates from primary S3WT and S3KO MEFs and DFs, exposed to TGF-β1 for the indicated time periods. Smad3 expression was reintroduced in S3KO cells by adenoviral infection at MOI 40. Ethidium bromide-stained 18S rRNA bands are shown to control for RNA loading.
Figure 5: Effect of TGF-β1 on extracellular matrix synthesis in S2WT, S2KO, S3WT, and S3KO fibroblasts. A) TGF-β1-induced fibronectin synthesis in the different fibroblasts. Cells were serum-starved in the presence of indicated concentrations TGF-β1 for 18h, after which cells were metabolically labeled for 4h with [35S]methionine in the presence of indicated concentrations TGF-β1. Conditioned medium was collected and fibronectin was affinity-purified using gelatin-sepharose beads, followed by separation on 6% SDS-polyacrylamide gels. A 72 kDa protein, corresponding to MMP-2, was co-purified. B) Identification of the 72 kDa TGF-β1-induced gelatin-sepharose binding protein as matrix-metalloproteinase MMP-2. MMP-2 protein was induced by TGF-β1 as assessed by zymography, metabolic labeling, as well as MMP-2 Western blot analysis, using gelatin-sepharose-purified conditioned medium from fibroblasts as described above. C) TGF-β1-induced PAI-1 production in S2WT, S2KO, S3WT, and S3KO fibroblasts, as well as in primary S3WT and S3KO MEFs and DFs. Smad3 expression was reintroduced in S3KO cells by adenoviral infection at MOI 40, and adenoviral β-galactosidase (β-gal) infection at MOI 40 was done in parallel as control. Cells were treated with indicated concentrations TGF-β1 for 5h and [35S]methionine was added during the last 3h. Extracellular matrix proteins were extracted and separated on 8% SDS-polyacrylamide gels. Experiments were performed at least three times and representative results are presented.

Figure 6: Activation of TGF-β-responsive reporters in S2WT, S2KO, S3WT, and S3KO fibroblasts. The effect of TGF-β1 on activation of the 3TP-Lux reporter (A,B), the (SBE)4-luciferase reporter (C,D), or the ARE-luciferase reporter (E,F) was studied in S2WT versus S2KO fibroblasts (A,C,E), and in S3WT versus S3KO fibroblasts (B,D,F). FAST-1 was co-
expressed with the ARE-luciferase reporter. Reconstitution of Smad2 (A) or Smad3 (B, D) expression in the respective Smad-deficient fibroblasts resulted in recovery of induction of the 3TP-Lux reporter and (SBE)$_4$-luciferase reporter (white bars, control; gray bars, 20 pM TGF-β1; black bars, 80 pM TGF-β1). Experiments were performed at least three times and representative results are shown as the average of triplicate values, corrected for transfection efficiency as measured by β-galactosidase activity.
**Figure 1**

**A**

<table>
<thead>
<tr>
<th>Lung</th>
<th>Fibroblasts</th>
<th>Smad3Δex8</th>
<th>Smad3</th>
<th>Smad2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3WT</td>
<td>S3KO</td>
<td>Smad3Δex8</td>
<td>Smad3</td>
<td>Smad2</td>
</tr>
</tbody>
</table>

Smad3 →

**B**

![Graph showing luciferase activity](image)

Luciferase activity (arbitrary units)

Control, Smad2, Smad2+Smad3, Smad2+Smad3Δex8

**Figure 2**

![Graph showing CPM](image)

CPM

S2WT, S2KO, S3WT, S3KO
Figure 3

A

Time (hr)                          Smad2 fibroblasts       Smad3 fibroblasts
Genotype                           WT KO WT KO WT KO WT KO WT KO
p15                                
GAPDH                              

B

Time (hr)                          Smad2 fibroblasts       Smad3 fibroblasts
Genotype                           WT KO WT KO WT KO WT KO WT KO
p21                                
GAPDH                              

C

Time (hr)                          Smad2 fibroblasts       Smad3 fibroblasts
Genotype                           WT KO WT KO WT KO WT KO WT KO
p27                                
GAPDH                              

Relative expression p15/GAPDH
Relative expression p21/GAPDH
Relative expression p27/GAPDH
Figure 5

A

<table>
<thead>
<tr>
<th>Genotype</th>
<th>S2WT</th>
<th>S2KO</th>
<th>S3WT</th>
<th>S3KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 (pM)</td>
<td>0 20 80</td>
<td>0 20 80</td>
<td>0 20 80</td>
<td>0 20 80</td>
</tr>
</tbody>
</table>

Fibronectin

MMP-2

220 kDa

97 kDa

66 kDa

B

<table>
<thead>
<tr>
<th>Genotype</th>
<th>S2WT</th>
<th>S2KO</th>
<th>S3WT</th>
<th>S3KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 (pM)</td>
<td>0 80</td>
<td>0 80</td>
<td>TGF-β1 (pM)</td>
<td></td>
</tr>
</tbody>
</table>

Zymography

35S Metabolic labeling

MMP-2 Western

C

<table>
<thead>
<tr>
<th>MEF</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2KO</td>
<td>S3WT</td>
</tr>
<tr>
<td>0 20 80</td>
<td>0 20 80</td>
</tr>
</tbody>
</table>

TGF-β1 (pM)

PAI-1
Figure 6

A

3TP-Lux

Lucifase activity (arbitrary units)

S2WT  S2KO  S2WT  S2KO

B

3TP-Lux

Lucifase activity (arbitrary units)

S3WT  S3KO  S3WT  S3KO

C

(SBE)₅-Lux

Lucifase activity (arbitrary units)

S2WT  S2KO

D

(SBE)₅-Lux

Lucifase activity (arbitrary units)

S3WT  S3KO  S3WT  S3KO

E

ARE-Lux

Lucifase activity (arbitrary units)

S2WT  S2KO

F

ARE-Lux

Lucifase activity (arbitrary units)

S3WT  S3KO
Functional Characterization of TGF-β Signaling in Smad2- and Smad3-Deficient Fibroblasts
Ester Piek, WenJun Ju, Joerg Heyer, Diana Escalante-Alcalde, Colin L. Stewart, Michael Weinstein, Chuxia Deng, Raju Kucherlapati, Erwin P. Boettinger and Anita B. Roberts

J. Biol. Chem. published online March 21, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102382200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts