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Suppression of MafA-dependent transcription by transforming growth factor-β signaling

Haruka Matsumura [a], Takashi Kudo [a], Ayako Harada [a], Ritsuko Esaki [a], Hiroyuki Suzuki [b], Mitsuyasu Kato [b], Satoru Takahashi [a,*]

[a] *Department of Anatomy and Embryology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[b] *Department of Experimental Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*

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Abstract

MafA is a basic leucine zipper (b-Zip) type transcription factor that binds to the insulin promoter and regulates insulin transcription synergistically with Pdx-1 and NeuroD. Transforming growth factor-β (TGF-β) signaling has been reported to regulate activity of b-Zip transcription factor such as ATF-2 and acts as an important regulator of insulin gene transcription and pancreatic β cell maintenance. To investigate the relationship between MafA-dependent transcriptional activation and TGF-β signaling, we examined the effects of TGF-β signal on MafA-dependent transactivation of the rat insulin II gene promoter (RIPII-251) and a synthetic MafA-dependent promoter. MafA-dependent activation of the reporters was inhibited in the presence of Smad2/Smad4 or Smad3/Smad4 and a constitutively active TGF-β type I receptor and this inhibition was dependent upon the presence of MafA. Co-immunoprecipitation analyses revealed that MafA physically interacts with Smad2 or Smad3. These results suggest that MafA-dependent transcriptional activation is negatively regulated by TGF-β signaling.

Keywords: Large Maf; Smad; Transcription

MafA is a basic leucine zipper (b-Zip) transcription factor that regulates gene expression in both the lens and pancreas [1–5]. MafA recognizes a specific sequence, the Maf recognition element (MARE), which contains a cAMP responsive element (CRE) or a TPA responsive element (TRE), and *trans*-activates target genes containing this element within their regulatory sequences. Within the pancreas, MafA is exclusively expressed in β cells and is involved in insulin gene transcription together with several other transcription factors including Pdx-1 and NeuroD [6,7]. The expression of MafA within β cells is controlled by the glucose concentration [3,4]. Mice lacking MafA display reduced transcription of the insulin gene, intolerance to glucose, abnormal islet structure, and develop diabetes mellitus [8]. These reports indicate that MafA plays critical roles in insulin transcription, secretion, and β cell maintenance [6–9].

The transforming growth factor-β (TGF-β) super family is known to regulate a wide range of cellular functions, such as cell proliferation, differentiation, apoptosis, and morphogenesis [10–12]. Growing evidence suggests that TGF-β is involved in the regulation of insulin expression, secretion, and induction of apoptosis of β cells. For examples, TGF-β ligands, receptors, and R-Smads are expressed in adult rat pancreatic islets [13,14]. In fetal rat islets, activin receptor like kinase 7 (ALK7) is the most dominant type I receptor of the TGF-β superfamily [15] and ALK7 has been reported to induce apoptosis of β cells [16]. In addition, TGF-β has been reported to increase insulin release from pancreatic islets in a concentration-dependent

* Corresponding author. Fax: +81 29 853 6965.
E-mail address: satoruta@md.tsukuba.ac.jp (S. Takahashi).

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manner [17]. On the other hand, the Smad and TAK1 pathways in TGF-β signaling have been reported to regulate the transcriptional activity of ATF-2, which is a member of b-Zip type transcription factors and recognizes the CRE [18]. According to these observations, MafA function may be regulated by TGF-β signals.

To determine the relationship between MafA-dependent transcriptional activation and TGF-β signaling at the molecular level, we analyzed MafA-dependent transcriptional activation of the rat insulin II gene promoter (RIPII-251) and a MafA specific promoter in the presence or absence of TGF-β signals in NIH3T3 cells. We show that MafA-dependent transcriptional activation is inhibited by TGF-β signaling.

Materials and methods

Cell lines. Mouse fibroblast NIH3T3 and human embryonic kidney 293T cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid construction. Mouse MafA cDNA was subcloned into pFFX3-Flag [4] and the pcDNA3-6×Myc eukaryotic expression vector. Pdx-1 and NeuroD cDNAs were amplified by RT-PCR using MIN6 cDNA as template. The forward primer used for amplification of the Pdx-1 gene was 5′-ACCATGAACAGTGAGGAGCAGTAC-3′, and the reverse primer was 5′-GGTTAAGTTCCCTTATCCAGCTGC-3′. The forward primer used for NeuroD amplification was 5′-AACATGACCAAATCATACA GCGAG-3′ and the reverse primer was 5′-GTGAAACTGACGTGC CTCTAATCG-3′. The amplified fragments were completely sequenced and inserted into the pcDNA3.1+neo mammalian expression vector (Invitrogen). Expression constructs encoding Flag-Smads, ALK5TD, and Flag-c-Ski were constructed as described previously [19]. RIPII-251 and 6×cαCE2 promoters [20] were subcloned into pGL4- and pGL2-basic vector (Promega), respectively.

Transient transfection and luciferase assay. NIH3T3 cells were plated at a density of 2×10^5 cells/well on 12-well dishes and transfected for 24 h using Lipofectamine™ 2000 (Invitrogen) according to the manufacture's instructions. For each transfection, 150 ng of the luciferase reporter plasmid and 75 ng of MafA, Smad2, Smad3 and Smad4, and 50 ng of the ALK5 expression vectors were used. The total amount of transfected DNA was kept constant by adding empty vector, as needed. All plasmid DNA was prepared using the PureLink™ High Pure Plasmid Midiprep Kit (Invitrogen). Co-transfection of 75 ng of control pRL-tk plasmid was performed for normalization of transfection efficiency. Forty-eight hours later, total cell lysates were subjected to luciferase activity assessment, using the Dual-Luciferase reporter system (Promega). Measurements were performed with a GLOMAX 20/20 luminometer (Promega). Luciferase activities were normalized on the basis of *Renilla* luciferase expression from the pRL-tk control vector.

Immunoprecipitation and immunoblotting. 293T cells were plated at a density of 1×10^6 cells/well on 6-well dishes and transfected 24 h using Lipofectamine™ 2000 according to the manufacture's instructions. For each transfection, 1 μg of pcDNA3-6×Myc-MafA, 0.5 μg of Flag-Smads, and 0.5 μg of HA-ALK5TD expression vectors, were used. The total amount of transfected DNA was kept constant by adding pcDNA3.1+neo vector. Total cell lysates were solubilized in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 1.5% Trasyrol. The lysates were cleared and incubated with anti-Flag antibodies followed by incubation with Protein G-Sepharose beads (GE Healthcare UK Ltd.). The beads and antibodies were washed with lysis buffer, then the immunoprecipitates were eluted by boiling at 95 °C for 5 min in 1× SDS sample buffer containing 100 mM Tris–HCl (pH 8.8), 0.01% bromophenol blue, 36% glycerol, 4% SDS, 20 mM dithiothreitol. The immunoprecipitates and cell lysate control were subjected to SDS–PAGE (10% gel) and electro-transferred to Immobilon-P PVDF membranes (MILLPORE), and subjected to immunoblotting. Anti-Myc (9E10, Calbiochem), anti-hemaggulutinin (HA) (3F10, Roche), horseradish peroxidase (HRP)-anti-Flag M2 antibodies (Sigma) were used as primary antibodies. The reacted antibodies were detected using an ECL-plus Western Blotting Detection System (GE Healthcare UK Ltd.).

Results

TGF-β signals inhibit MafA-dependent transcription of the rat insulin II gene

MafA, Pdx-1, and NeuroD regulate insulin expression through binding to the insulin promoter in pancreatic β cells. To investigate the effect of TGF-β signal on transcriptional activation by these factors, we used the pGL-RIPII-251 reporter construct. As shown in Fig. 1A, this construct carries 251 bp ($-251 \sim -1$) of the rat insulin II gene promoter, which contains two Pdx-1 binding sites (A1 and A3), a NeuroD binding site (E1) and a MafA binding site (C1) driving expression of a luciferase reporter gene. We first examined the effect of MafA, Pdx-1, and NeuroD expression on RIPII-251 reporter activity in NIH3T3 cells, since these factors are expressed in β cell lines and it is thus difficult to estimate the individual contribution of these factors in these cells. As shown in Fig. 1B, RIPII-251 reporter activity was increased by MafA expression alone (4.6-fold), but not by Pdx-1 or NeuroD expression alone. Co-expression of MafA and NeuroD stimulated RIPII-251 activity (21.2-fold), whereas co-expression of MafA and Pdx-1 led to a level of reporter expression similar to that observed following MafA expression alone. Co-expression of Pdx-1 and NeuroD did not activate the RIPII-251 reporter. Furthermore, co-expression of all three factors stimulated reporter expression most strongly (49.7-fold). These results suggested that MafA is an important regulator of RIPII-251 reporter activity in NIH3T3 cells.

We next investigated the effect of TGF-β signaling on MafA-mediated activation of the RIPII-251 reporter activity. As shown in Fig. 1C, cells were transfected with RIPII-251 reporter plasmid along with plasmids expressing MafA, Pdx-1, and NeuroD, and various combinations of Smad2/Smad4, Smad3/Smad4, and ALK5TD (constitutively active TGF-β type I receptor) as indicated. Unexpectedly, RIPII-251 reporter activation induced by co-expression of MafA, Pdx-1, and NeuroD was inhibited by co-transfection of Smad2/Smad4 or Smad3/Smad4. Inhibition of RIPII-251 reporter activity by Smads was further inhibited in the presence of ALK5TD. The repressive effect of Smad3/Smad4 expression was greater than that of Smad2/Smad4 expression (6.3-fold versus 3.0-fold). These results demonstrate that *trans*-activation of the RIPII-251 reporter by MafA, Pdx-1, and NeuroD is suppressed by co-expression of factors that mediate TGF-β signals.

To clarify the molecular mechanism of suppression of the reporter promoter in NIH3T3 cells by factors that mediate TGF-β signals, we next evaluated reporter expres-

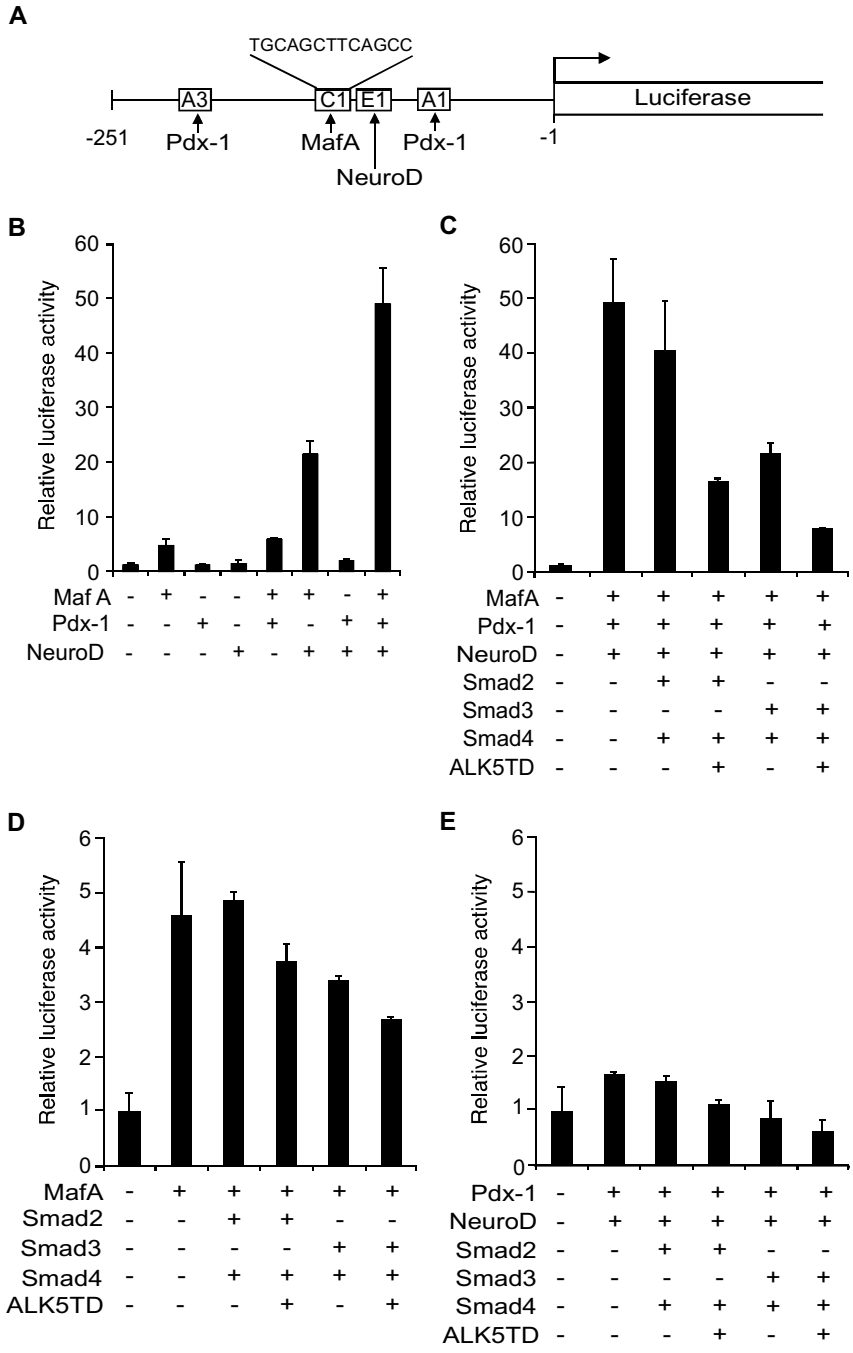

Fig. 1. Smads inhibit activation of the rat insulin II gene promoter by MafA, Pdx-1, and NeuroD. (A) A schematic representation of the rat insulin II gene promoter reporter plasmid (pGL-RIPII-251). The location of the Pdx-1 (A3, −216/−207; A1, −81/−77), NeuroD (E1, −100/−91), and MafA (C1, −115/−107) binding elements are shown in the rat insulin II gene promoter (−251/−1). (B–E) NIH3T3 cells were transiently co-transfected with MafA, Pdx-1, and NeuroD expression plasmids and the pGL-RIPII-251 reporter plasmid (B). Analysis of the effects of expression of MafA, Pdx-1, and/or NeuroD on reporter expression. Analysis of the effects of Smad2, Smad3, Smad4, and/or ALK5TD co-expression on reporter gene expression in the presence of MafA, Pdx-1, and NeuroD (C), MafA alone (D), or Pdx-1/NeuroD alone (E). Co-transfection of control pRL-tk plasmid was performed for normalization of transfection efficiency. Luciferase activities were measured 48 h after transfection. Error bars mean ± SD.

sion following coexpression of Smad2/Smad4, Smad3/Smad4, and ALK5TD in the presence of only MafA. Expression of MafA alone stimulated luciferase activity about 4-fold (Fig. 1D). MafA-mediated transcriptional activation was inhibited by co-transfected with Smad3/Smad4 and ALK5TD. The inhibitory effect of Smad2/Smad4 and ALK5TD was weaker than that of Smad3, Smad4, and ALK5TD co-expression. We observed little effect of Smad2/Smad4, Smad3/Smad4, and ALK5TD co-expression on reporter expression in cells co-transfected with Pdx-1 and NeuroD (Fig. 1E). These results indicated that TGF-β signals inhibit the transcriptional activation

of the RIPII-251 reporter following MafA, Pdx-1, and NeuroD co-expression through an effect upon MafA.

Smads inhibit the activation of 6×cαCE2 (αA-crystallin gene) promoter by MafA

There are two possible mechanisms through which TGF-β signals might inhibit MafA transcriptional activity. One possibility is that Smads bind to the RIPII-251 promoter sequences directly and inhibit MafA-dependent reporter activation. The second possibility is that Smads form a complex with MafA and inhibit its activity directly. To evaluate these two possibilities, we examined the effect of TGF-β signals on another MafA-dependent promoter; the 6×cαCE2 promoter derived from the chicken αA-crystallin promoter (Fig. 2A) [20]. As shown in Fig. 2B, co-expression of Smad3, Smad4, and ALK5TD inhibited MafA-dependent activation of the 6×cαCE2 promoter to an extent similar to their effect upon the RIPII-251 reporter, as shown in Fig. 1. Co-expression of Smad3, Smad4, and ALK5TD in absence of MafA expression had no effect upon the basal activity of the reporter. This result indicates that inhibition of MafA-dependent transcription by Smads is not specific to the RIPII-251 reporter. Both the RIPII-251 and 6×cαCE2 promoters contain MAREs, whereas neither the 6×cαCE2 sequence nor the β-actin basal promoter contain CAGA sequences, which are Smad binding elements. Therefore, these results suggest that since Smads do not bind directly to the 6×cαCE2 or β-actin basal promoters, the TGF-β signal must act directly upon MafA to repress transcriptional activity of this reporter.

MafA physically interacts with Smad proteins

Smads are known to interact with many transcription factors and to regulate their transcription activity [12,21]. We next examined whether MafA directly interacts with Smad proteins by co-immunoprecipitation analyses. 293T cells were co-transfected with plasmids encoding 6×Myc-tagged MafA, Flag-tagged Smads (Smad2, Smad3, and Smad4) and HA-tagged ALK5TD. Cell lysates prepared from the transfected cells were immunoprecipitated with an anti-Flag antibody, followed by Western blot analysis using an anti-Flag antibody to detect Flag-tagged Smad2, Smad3, and Smad4, an anti-Myc antibody to detect 6×Myc-tagged MafA, or an anti-HA antibody to detect HA-tagged ALK5TD (Fig. 3). The results indicated that MafA interacts with Smad2, Smad3, and Smad4. The inter-

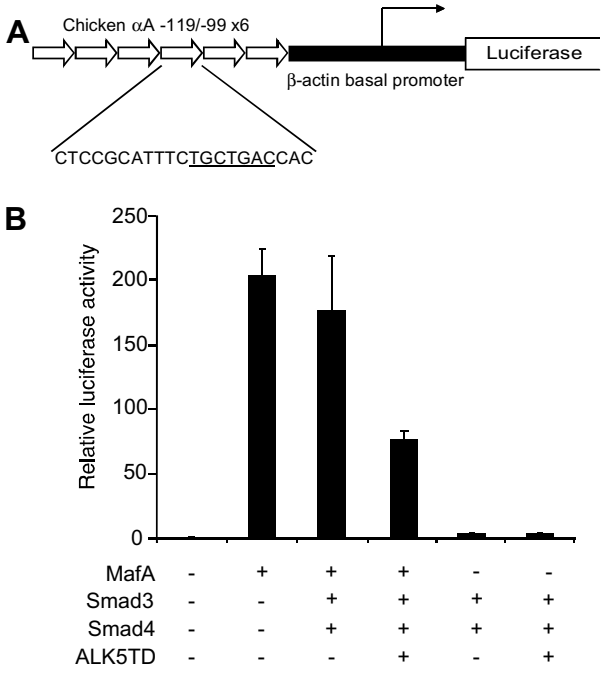

Fig. 2. Smads inhibit activation of the 6×cαCE2 promoter by MafA. (A) A schematic representation of the 6×cαCE2 reporter plasmid (pGL-6×cαCE2). Six tandem copies of the αCE2 sequence (−119/−99) of the chicken αA crystalline promoter and a chicken β-actin basal promoter were inserted into the pGL2 luciferase reporter plasmid. The half MARE sequence is underlined in the αCE2 sequence. (B) NIH3T3 cells were transiently transfected with pGL-6×cαCE2 and MafA expression plasmids. Smad3, Smad4, and ALK5TD expression plasmids were co-transfected as indicated. Co-transfection of pRL-tk plasmid was performed for normalization of transfection efficiency. Luciferase activities were measured 48 h after transfection. Error bars mean ± SD.

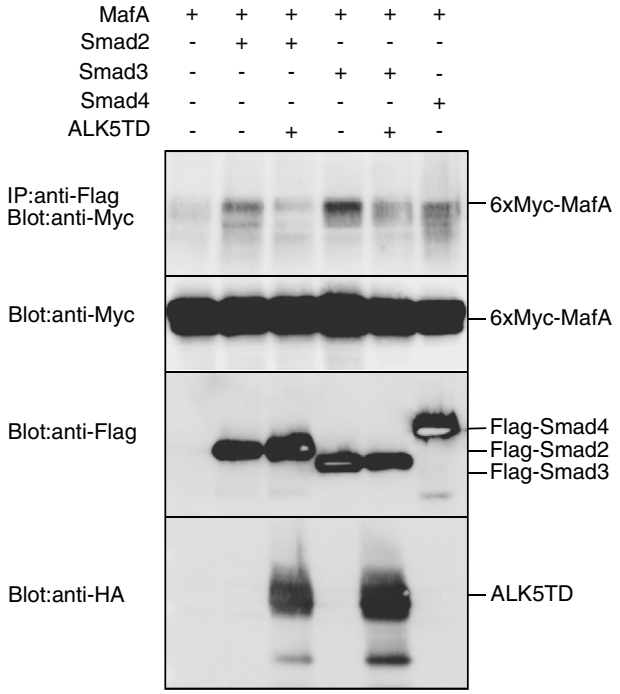

Fig. 3. Immunoprecipitation and Western blot analysis of the interaction of MafA with Smad proteins. 6×Myc-tagged MafA, Flag-tagged Smad2, Smad3, Smad4, and HA-tagged ALK5TD were co-expressed in 293T cells as indicated in the top panel. The cell lysate was immunoprecipitated (IP) with an anti-Flag antibody, followed by Western blot analysis (Blot) with an anti-Flag antibody (to detect Flag-tagged Smad2, Smad3, and Smad4), an anti-Myc antibody (to detect 6×Myc-tagged MafA), or an anti-HA antibody (to detect HA-tagged ALK5TD), as indicated in the lower panels.

actions between Smads and MafA were detected in the absence but not in the presence of co-expressed ALK5TD. These results show that Smads directly bind to MafA.

Discussion

The insulin promoter contains three indispensable elements for β cell-specific activity: the A3, C1, and E1 elements recognized by Pdx-1, MafA, and NeuroD, respectively (Fig. 1A). We previously reported the generation and analyses of MafA-deficient mice [8]. MafA-deficient mice show normal pancreatic islet development at birth, but exhibit decreased insulin gene transcription, an increase in the α/β cell ratio, abnormal islet architecture and abnormal glucose tolerance after several weeks. A fraction of these mice subsequently develop overt diabetes mellitus. These results clearly indicate that MafA is a key regulator of β cell function and maintenance. In addition, TGF-β stimulates insulin secretion and increases the expression of the insulin mRNA in a concentration-dependent manner in islet cells and β cell lines [17,22,23]. As such, we hypothesized that TGF-β signaling might stimulate MafA-dependent transactivation of an insulin reporter gene in NIH3T3 cells. Contrary to our expectations, we found that TGF-β signals strongly inhibited transactivation of an insulin promoter reporter construct by MafA. Moreover, we detected direct protein–protein interaction of Smads with MafA. This interaction between Smads and MafA has not been previously identified and suggests a mechanism for cross-talk between these signaling pathways.

TGF-β has been reported to stimulate Pdx-1-mediated transactivation of insulin gene transcription [23]. According to this study, TGF-β increased binding of Pdx-1 to the A3 element within the insulin promoter based on an EMSA performed using cell lysates prepared from the INS-1 cell line. In contrast, a similar analysis revealed that binding of MafA to element was unchanged by TGF-β signaling. We have shown here that Smads inhibit the transcriptional activity of MafA in NIH3T3 cells. Taken together, we conclude that TGF-β increases Pdx-1 transcriptional activity and decreases MafA transcriptional activity depending upon the particular cell and environmental condition.

We have demonstrated here that the activation of a luciferase reporter gene by MafA is suppressed by TGF-β signals and that this suppression is more prominent in the presence of ALK5TD. However, the interaction between MafA and Smads appears to be independent of ALK5TD. To identify the molecular mechanism of this suppression, EMSA was performed the absence or presence of ALK5TD, but there was no significant difference between the DNA binding activity of MafA under these conditions (data not shown). These results suggest that the activated type I receptor may recruit a suppressor complex such as Ski/Sno and change components of transcriptional complex to inhibit transcriptional activation mediated by MafA. Further analysis is needed to identify the molecular mechanism of the suppression of MafA-mediated transcriptional transactivation by ALK5TD.

The physiological role of the suppression of MafA-mediated transcriptional transactivation by TGF-β signaling is unclear at this time. One possibility is that TGF-β signaling might regulate apoptosis of β cells through an effect upon MafA activity. As previously demonstrated, ALK7 is the dominant type I receptor of the TGF-β superfamily receptor in β cells and ALK7 induces apoptosis of these cells [15,16]. The activation of ALK7 increases Smad2 phosphorylation, reduces protein kinase B (Akt) activity and is associated with increased levels of the bioactive forms of caspase-3. Since MafA deficiency induces abnormal islet structure and decreases the ratio of α/β cells, suppression of MafA function may play a role in the induction of apoptosis by ALK7. Further experiments are necessary to reveal the effect of TGF-β signaling on MafA function *in vivo*.

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