Sertad1 encodes a novel transcriptional co-activator of SMAD1 in mouse embryonic hearts

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ABSTRACT

Despite considerable advances in surgical repairing procedures, congenital heart diseases (CHDs) remain the leading noninfectious cause of infant morbidity and mortality. Understanding the molecular/genetic mechanisms underlying normal cardiogenesis will provide essential information for the development of novel diagnostic and therapeutic strategies against CHDs. BMP signaling plays complex roles in multiple cardiogenic processes in mammals. SMAD1 is a canonical nuclear mediator of BMP signaling, the activity of which is critically regulated through its interaction partners. We screened a mouse embryonic heart yeast two-hybrid library using *Smad1* as bait and identified SERTAD1 as a novel interaction partner of SMAD1. SERTAD1 contains multiple potential functional domains, including two partially overlapping transactivation domains at the C terminus. The SERTAD1-SMAD1 interaction *in vitro* and in mammalian cells was further confirmed through biochemical assays. The expression of *Sertad1* in developing hearts was demonstrated using RT-PCR, western blotting and *in situ* hybridization analyses. We also showed that SERTAD1 was localized in both the cytoplasm and nucleus of immortalized cardiomyocytes and primary embryonic cardiomyocyte cultures. The overexpression of SERTAD1 in cardiomyocytes not only enhanced the activity of two BMP reporters in a dose-dependent manner but also increased the expression of several known BMP/SMAD regulatory targets. Therefore, these data suggest that SERTAD1 acts as a SMAD1 transcriptional co-activator to promote the expression of BMP target genes during mouse cardiogenesis.

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1. Introduction

Congenital heart diseases (CHDs) occur in as many as 1–5% of newborns and remain the leading noninfectious cause of infant morbidity and mortality [1,2]. Understanding the molecular and genetic mechanisms governing normal cardiogenesis will provide critical information for the development of novel diagnostic and therapeutic strategies against CHDs.

Bone Morphogenetic Proteins (BMPs) play complex roles during mammalian heart development; mutations that disturb BMP signaling lead to various forms of CHDs in both animal models and human patients [3–6]. BMP signaling is transduced through heterodimeric complexes of type I and type II serine/threonine kinase receptors on the cell surface. After formation of the receptor–ligand complex, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates a group of cytoplasmic proteins called BMP receptor-activated-SMADs (R-SMADs), including SMAD1, SMAD5, and SMAD8. Phosphorylated SMAD1/5/8 associates with SMAD4, a transcriptional co-activator of R-SMADs, and the SMAD complex translocates to the nucleus to regulate the expression of target genes [7,8]. To activate the transcription of target genes, BMP R-Smads form a large nucleoprotein complex with SMAD-binding DNA elements, sequence specific transcription factors, and transcriptional co-activators. Many cardiogenic genes, such as *Nkx2.5* [9–12], are directly regulated through BMP R-SMADs.

It has long been hypothesized that SMAD4 is essential for nearly all R-SMAD-mediated transcription [13,14]; however, this idea has been challenged by later studies. The inactivation of *Smad4* in epiblasts causes embryonic lethality at E8.5, yet heart rudiments are still formed in mutants, indicating that *Smad4* is not required for the *Bmpr1a*-mediated induction of cardiomyocytes from the lateral mesoderm [15,16]. We recently showed that *Smad4* promotes cardiomyocyte proliferation and survival through the upregulation of *Mycn* expression; however, many BMP-mediated processes, including AV canal specification, extracellular matrix deposition, and outflow tract remodeling are not compromised by the myocar-

dial inactivation of *Smad4* [17]. These results collectively suggest the presence of other co-activators of BMP R-SMADs that are also crucial for mediating the complex activities of BMP signaling during cardiogenesis.

In this study, we report the identification of SERTAD1 as a novel interaction partner of SMAD1 in mouse embryonic hearts. *Sertad1* encodes a 236-a.a. protein with five potential functional domains [18–21]. The N terminus of SERTAD1 is homologous to the Cyclin A binding sequence (a.a.1–30); however, whether Sertad1 interacts with Cyclin A has not been experimentally determined. The SERTA domain (a.a.43–82) refers to a novel heptad hydrophobic repeat, which has been identified in multiple proteins from insects to humans. The exact biological activities of the SERTA domain remain unclear. The CDK4 binding domain (a.a.30–160) is responsible for the direct interaction between CDK4 and SERTAD1. The C-terminal half of SERTAD1 contains two partially overlapping transactivation domains: the PHD-bromodomain-interacting domain (a.a.161–178), which interacts with the bromodomain and/or PHD zinc finger-containing general transcriptional co-activators (such as p300/CBP), and the acidic region (a.a.167–220), which has intrinsic transactivation activity [18–20]. SERTAD1 does not contain any known DNA binding motifs, nor has this protein been reported to interact with DNA directly. The current view is that SERTAD1 forms a complex with transcription factors (such as E2F1) to co-activate the transcription of target genes [18,19]. In addition to regulating gene expression, SERTAD1 directly interacts with CDK4 to antagonize the activity of the cdk inhibitor p16INK4, thereby promoting cell proliferation [21,22]. Our study provides the first evidence that SERTAD1 acts as a transcriptional co-activator of SMAD1 to promote the expression of BMP target genes in mouse embryonic hearts.

2. Materials and methods

2.1. Mouse maintenance

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All protocols were approved through the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. The CD1 mice were purchased from Charles River.

2.2. Detection of the SERTAD1–SMAD1 interaction

Yeast two-hybrid screening using *Smad1* as bait, *in vitro* GST-pull down, and mammalian cell GST-pull down analyses were performed as previously described [23,24].

2.3. Seartad1 expression analysis

Total RNA from pooled embryonic hearts or whole embryonic bodies was isolated using the RNeasy mini kit (Qiagen). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis was performed using the OneStep RT-PCR kit (Qiagen). Western analysis was performed as previously described [24]. Non-isotope section *in situ* analysis was performed as previously described [25]. The ~300 bp-cDNA fragment from the 3′ untranslated region of *Sertad1* was used as the probe.

2.4. Cell culture, SERTAD1 subcellular localization analysis and reporter analysis

NkL-TAg cells were cultured as previously described [26]. Primary cardiomyocytes were isolated from E13.5 embryos as previously described [27]. BMP4 was purchased from R&D. For immunostaining assays, the cells were cultured on glass coverslips and incubated with a primary antibody at 4 °C overnight. The next day, the cells were incubated with an Alexa488- (or Alexa594-) conjugated secondary antibody (Life Technologies). The cells were further stained with DAPI to visualize total nuclei. The samples were examined using a Zeiss LSM 710 confocal microscope. Nuclear/cytoplasmic fractionation was achieved using a kit purchased from ThermoSceintific following manufacturer's instructions. Western analysis was performed using antibodies against SERTAD1, MEK1/2 (a cytoplasmic marker) and LSD1 (a nuclear marker). For the reporter analysis, NkL-TAg cells were co-transfected with a reporter construct and different doses of a construct expressing SERTAD1 using Lipofectamine 2000 (Life Technologies). The luciferase activity was determined using the Luciferase Assay kit from Promega. A lacZ reporter plasmid was co-transfected to normalize the transfection efficiency. LacZ activity was measured using the Beta-Glo Assay System (Promega).

2.5. Primary antibodies used in this study

The HA antibody was purchased from Babco. The GST antibody was purchased from Amersham. The SERTAD1 antibody was obtained from AVIVA. Antibodies against phosphorylated SMAD1 (p-SMAD1), MEK1/2, LSD and the myc tag were purchased from Cell Signaling. Antibodies against TBX20 and ID2 were purchased from Sigma. Antibodies against ACTIN, TUBULIN and cardiac troponin T (cTnt) were purchased from the Iowa Hybridoma Bank.

3. Results

3.1. Identification of SERTAD1 as a novel interaction partner of SMAD1 in mouse embryonic hearts

To identify novel interaction partners of BMP R-SMADs during cardiogenesis, we screened a mouse embryonic heart two-hybrid library [24] using *Smad1* as bait. We acquired three identical clones that align to the region at −36 to + 841 bp (relative to the start codon) of the *Sertad1* cDNA. This fragment covers the entire coding region of *Sertad1* (+1 to +708 bp). Fig. 1A shows that yeast cells harboring both the *Sertad1* prey construct and the *Smad1* bait construct grew on the selective medium, indicating a positive interaction between SERTAD1 and SMAD1. In the control experiment, the *Sertad1* prey did not interact with the empty bait vector. To exclude the possibility that the SERTAD1-SMAD1 interaction was mediated through unknown yeast proteins, we performed an *in vitro* GST pull-down analysis (Fig. 1B). The ^{35}S labeled SERTAD1 was pulled down with GST-SMAD1 but not with GST, demonstrating a direct interaction between the two proteins. We further performed a mammalian cell GST pull-down analysis to examine the interaction of these proteins in mammalian cells. GST-Sertad1 (or GST alone, negative control) and HA-Smad1 were co-expressed in HEK293 cells. A constitutively active form of ALK6 (Q203D) was co-transfected into HEK cells to activate the BMP intracellular signaling cascade. HA-Smad1 was co-purified with GST-Sertad1, but not with GST, from cell lysates (Fig. 1C), confirming that SERTAD1 interacts with SMAD1 in mammalian cells. In the absence of constitutively active ALK6, the association of SMAD1 with SERTAD1 was marginal (data not shown), indicating that the SMAD1-SERTAD1 interaction is enhanced through active BMP signaling. We further confirmed that active (phosphorylated) SMAD1 was co-purified with SERTAD1 through western analysis using a p-SMAD1 specific antibody (Fig. 1D). In both *in vitro* and *in vivo* pull down assays, the cDNA fragment encoding the full ORF of SERTAD1 (+1 to +708 bp) was used. Therefore, the interaction between the two
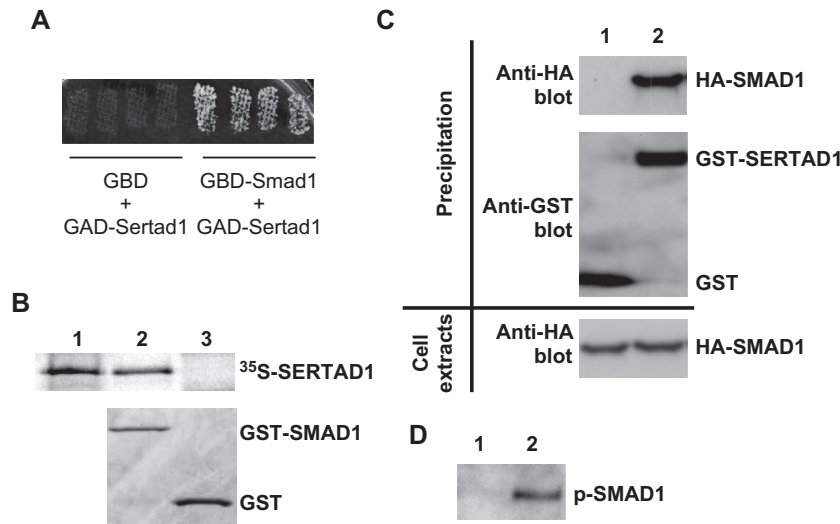
Fig. 1. SERTAD1 interacts with SMAD1. (A) AH109 yeast cells were transformed with various constructs as indicated and grown on selective medium. Growth indicates a positive interaction between the bait and prey. GBD: empty bait vector; GBD-Smad1: bait vector containing the full length of *Smad1*; GAD-Sertad1: the prey vector containing *Sertad1*. (B) SERTAD1 was labeled with [^{35}S]methionine and incubated with bacterial-expressed GST (negative control) or GST-SMAD1. The signals were visualized through autoradiography (top panel). The bottom panel shows the Coomassie blue staining of a SDS–PAGE gel. Lane 1: free probe (10%); lane 2: ^{35}S-SERTAD1 + GST-SMAD1; lane 3: ^{35}S-SERTAD1 + GST. (C) A plasmid expressing HA-SMAD1 was co-transfected with a plasmid expressing GST alone (negative control, lane 1) or GST-SERTAD1 (lane 2) into HEK293 cells. The expression of HA-SMAD1 in HEK cells was confirmed through western analysis using an anti-HA antibody (bottom panel). The GST and GST-SERTAD fusion proteins were purified with GST-binding beads followed by western blot analysis. An HA antibody was applied to determine whether HA-SMAD1 was co-purified with GST-CHD7 (top panel). The successful purification of GST and GST-SERTAD1 was confirmed through western analysis using a GST antibody (middle panel). Lane 1: GST + HA-SMAD1; lane 2: GST-SERTAD1 + HA-SMAD1. (D) The leftover samples from the experiment shown in panel C were applied for an additional western analysis using a p-SMAD1-specific antibody. The active form of SMAD1 was co-purified with GST-SERTAD1. Lane 1: GST + HA-SMAD1; lane 2: GST-SERTAD1 + HA-SMAD1.

proteins is not mediated through the extra 12 amino acids at the N-terminus of the protein encoded by the original clone identified from two-hybrid screening.

3.2. Sertad1 is expressed in embryonic hearts

To directly examine whether *Sertad1* is expressed during mouse cardiogenesis, we first performed RT-PCR analysis using RNA samples extracted from pooled embryonic hearts (E9.5–E11.5) or embryonic bodies. We detected the expression of *Sertad1* and its homolog *Sertad2* in embryonic hearts, whereas the cardiac expression of the other two *Sertad1* homologs, *Sertad3* and *Cdca4*, was not detected (Fig. 2A). To examine SERTAD1 expression at the protein level, we performed western analysis. The expression of SERTAD1 was detected in embryonic hearts at all stages tested (E10.5–E15.5) (Fig. 2B). The expression level was reduced at E15.5 compared with earlier stages. The expression of *Sertad1* in embryonic hearts was further verified through *in situ* hybridization analysis (Fig. 2C, C′). In embryonic hearts at E10.5, *Sertad1* transcripts were observed only in the myocardium, and not in the endocardial or cushion mesenchymal cells, suggesting that at this stage, *Sertad1* primarily regulates the cardiogenic processes in myocardial cells, but not in endocardial/mesenchymal cells.

3.3. SERTAD1 is localized in both the cytoplasm and nucleus of cardiomyocytes

Depending on the cell types examined, SERTAD1 has been reported as localized to the nucleus [20,22,28] or cytoplasm [29]. We therefore determined the subcellular localization of SERTAD1 in cardiomyocytes. NkL-TAg cells are immortalized cardiomyocytes widely used to study various biological processes in heart muscle cells [26,30]. We immunostained NkL-TAg cells using a SERTAD1-specific antibody and detected positive signals in both the nucleus and cytoplasm (Fig. 3A). The subcellular localization of SERTAD1 was not altered by BMP stimulation. The western analysis confirmed that SERTAD1 is detected in both the cytoplasmic

and nuclear fractions of NkL-TAg cells (Fig. 3B). The subcellular localization of SERTAD1 was further confirmed through immunostaining primary embryonic cardiomyocyte cultures (E13.5) (Fig. 3C). Although the signal detected in the cytoplasm was stronger, positive signals were clearly detected in nuclei.

3.4. SERTAD1 enhances BMP/SMAD-mediated transcription

Considering the function of SERTAD1 as a transcriptional co-activator of E2F1 [18,19], we examined whether *Sertad1* enhances BMP/SMAD-mediated transcription. We first performed reporter analyses using two BMP-responsive reporters in NkL-TAg cells. The SBE-lux construct contains 4 copies of SMAD binding elements from the *JunB* promoter and has been widely used to study the transcriptional regulation of TGFβ/BMP signaling [31,32]. In the Nkx2.5-lux(epi) construct, the genomic DNA containing the G/S and AR2 enhancers of *Nkx2.5* was fused with the SV40 minimum promoter and cloned into the pREP4-luc reporter vector. The G/S and AR2 enhancers of *Nkx2.5* harbor functional BMP R-SMAD binding elements demonstrated through *in vitro* and transgenic studies [9,10]. pREP4-luc is an Epstein–Barr virus-based episomal vector that undergoes chromatinization after transfected into mammalian cells [33]. Therefore, the Nkx2.5-lux(epi) construct is expected to more closely resemble activities of enhancers in the context of chromosomes. Overexpression of SERTAD1 enhanced the activities of both reporters in a dose-dependent manner (Fig. 4A and B). We further showed that the overexpression of SERTAD1 upregulated the expression of endogenous NKX2.5, ID2 and TBX20 upon BMP4 stimulation in NkL-TAg cells (Fig. 4C). *Nkx2.5*, *Id2*, and *Tbx20* are direct regulatory targets of BMP R-SMADs in embryonic hearts.

4. Discussion

The activities of R-SMADs are critically regulated through their interaction partners [7,8]. The present study provides convincing
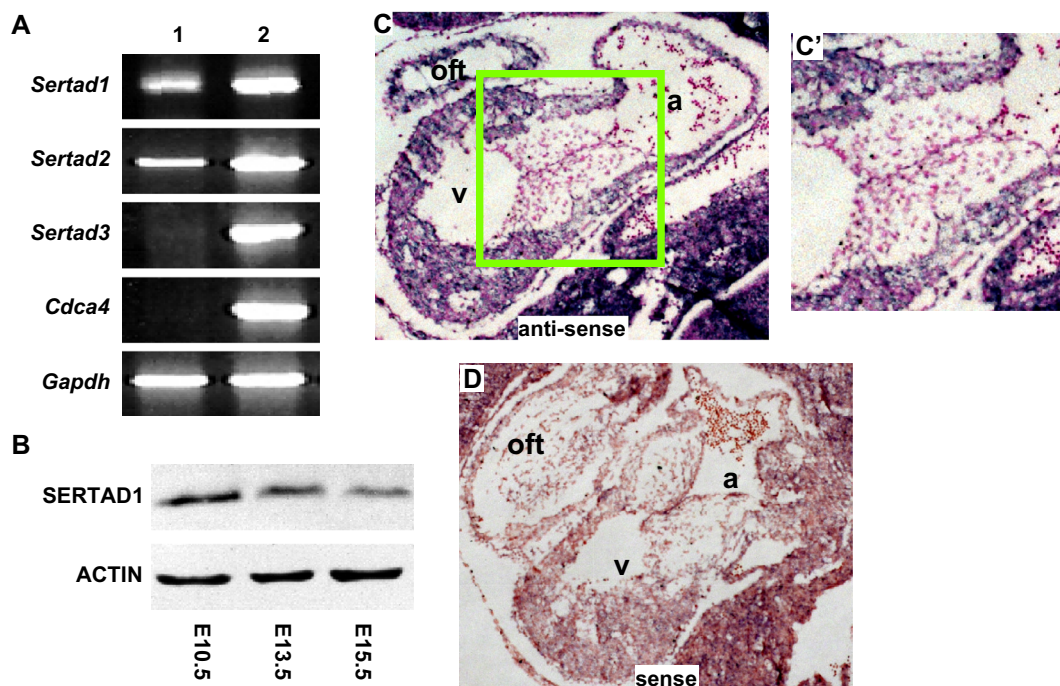
Fig. 2. *Sertad1* is expressed in developing mouse hearts. (A) RT–PCR analysis was performed using RNA samples extracted from pooled E9.5–11.5 embryonic hearts (lane 1) or whole embryos (lane 2). No signal was detected from the "no RT" control reactions (data not shown). *Gapdh* was used as a loading control. (B) Western analysis was performed on proteins extracted from embryonic hearts using anti-SERTAD1 or anti-ACTIN antibodies (loading control). (C, C′) *In situ* hybridization analysis was performed on sagittal sections of E10.5 embryos using a *Sertad1* anti-sense probe (purple). Total nuclei were visualized with nuclear-fast-red staining (red). (C′) corresponds to the boxed area of panel C. (D) The sense probe was used as a negative control. a: atrium; oft: outflow tract; v: ventricle (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
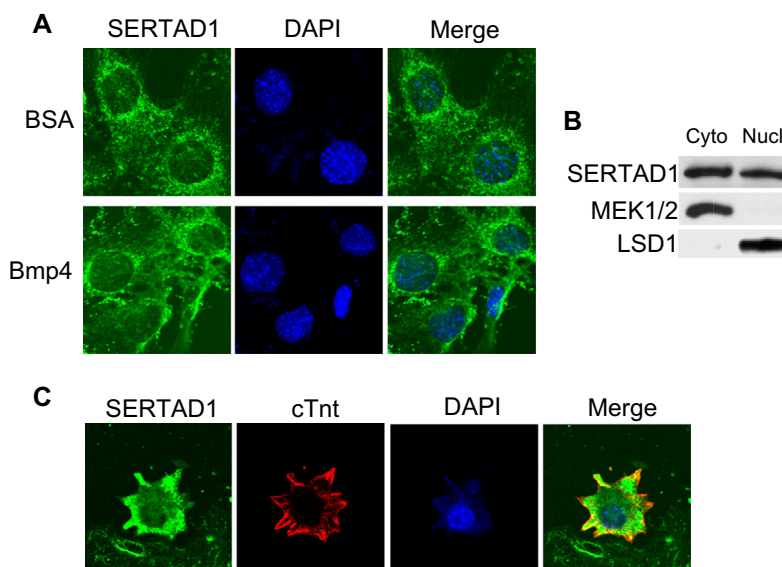

Fig. 3. SERTAD1 is localized in both the cytoplasm and nucleus of cardiomyocytes. (A) NkL-TAg cells were treated with BSA (negative control) or 100 ng/ml BMP4 for 2 h, followed by immunostaining using an anti-SERTAD1 antibody (green). The nuclei were visualized with DAPI staining (blue). The samples were examined under a Zeiss confocal microscope. Green signals were detected in both the cytoplasm and nucleus. No difference was observed between the BSA- and BMP4-treated samples. (B) The cytoplasmic and nuclear fractions of NkL-TAg cells were subjected to western analysis using a SERTAD1 antibody. MEK1/2 and LSD1 were used as cytoplasmic and nuclear controls, respectively. (C) Embryonic cardiomyocytes (E13.5) were isolated and cultured for 24 h. Immunostaining was performed using antibodies against SERTAD1 (green) and cTnt (red). Total nuclei were visualized with DAPI staining (blue). Green signals were detected in both the cytoplasm and nucleus (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

evidence supporting the idea that SERTAD1 acts as a co-activator of SMAD1 to promote BMP-mediated gene expression in mouse embryonic hearts. The SERTAD1-SMAD1 interaction is demonstrated with multiple complementary approaches, including yeast two-hybrid, *in vitro* GST pull-down and mammalian cell co-purifi-

cation analyses. Our sub-cellular localization analysis showed that SERTAD1 was localized in both the cytoplasm and nucleus in cardiomyocytes. Consistent with the presence of SERTAD1 in the nucleus, we showed that activated (phosphorylated) SMAD1, localized in the nucleus, was co-purified with SERTAD1 from mamma-
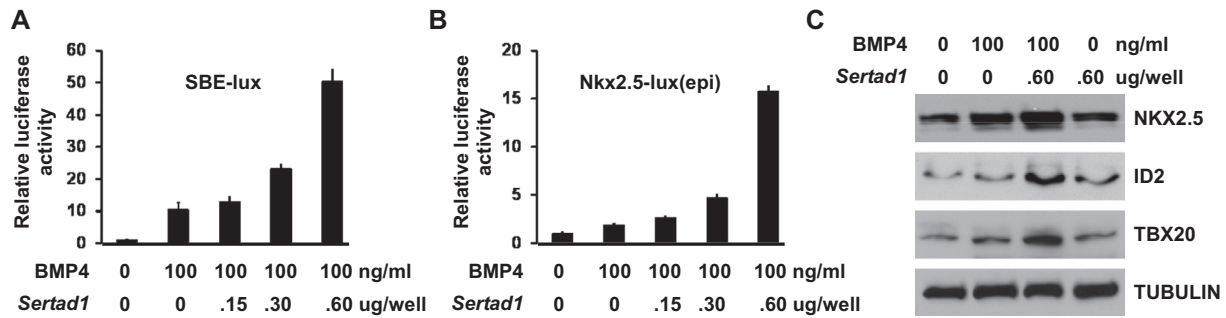
Fig. 4. Overexpression of SERTAD1 enhanced BMP R-SMAD mediated transcription in cardiomyocytes. (A) NkL-TAg cells were cultured in 24-well plates and were co-transfected with SBE-lux and different doses of the construct expressing SERTAD1. The cells were treated with or without BMP4 (100 ng/ml) for 48 h, followed by luciferase analysis. The luciferase activity from cells that were not transfected with the *Sertad1* construct and were not treated with BMP4 was set at 1.0. The data were averaged from three independent experiments. The error bars represent standard deviation. (B) Same as panel A except that the Nkx2.5-lux(epi) reporter construct was used. (C) NkL-TAg cells were transfected with an empty vector or a plasmid expressing SERTAD1 and treated with or without BMP4 (100 ng/ml) for 48 h. Total protein extracts were subjected to western analysis using antibodies as indicated. The overexpression of SERTAD1 upregulated the expression of the three BMP targeted genes. TUBULIN was used as a loading control.

lian cells. The nuclear localization of SERTAD1 and its interaction with p-SMAD supports SERTAD1 as a transcriptional co-activator. Furthermore, our functional analysis showed that the overexpression of SERTAD1 in immortalized cardiomyocytes not only enhanced the activities of two BMP-responsive reporters but also upregulated the expression of endogenous NKX2.5, ID2 and TBX20 upon BMP stimulation.

In a previous study, we showed that the inactivation of *Smad4* in the myocardium of embryonic hearts led to severe myocardial wall defects and embryonic lethality [17], supporting a critical role for SMAD-mediated transcription during cardiomyogenesis. Notably, many cardiogenic activities of BMP signaling were not impaired by the deletion of *Smad4*. For example, *Nkx2.5* and some other known BMP/SMAD target cardiogenic genes remain normally expressed in *Smad4*-deleted hearts [17]. As a novel SMAD1 co-transcriptional activator, we speculate that SERTAD1 mediates certain SMAD4-independent cardiogenic activities of BMP signaling. Consistently, we observed that the overexpression of SERTAD1 upregulated NKX2.5 expression. We are currently examining the interrelationship between SERTAD1 and SMAD4.

The subcellular localization of SERTAD1 is currently under debate. The nuclear localization of SERTAD1 has been reported in multiple studies [20,22,28]. However, a recent study using an anti-SERTAD1 antibody showed that endogenous SERTAD1 is exclusively localized in the cytoplasm of primary fibroblasts and some cancer cell lines [29]. These authors argued that the differential results reflect the use of tagged versions (HA or Flag tag) of SERTAD1 to examine its cellular localization. They speculated that tagging SERTAD1 somehow altered the distribution of the protein in cells. To avoid any potential complication from tagging SERTAD1, we used a SERTAD1-specific antibody to examine the subcellular distribution of endogenous SERTAD1. In a cardiomyocyte cell line and primarily cultured embryonic cardiomyocytes, SERTAD1 is localized in both the cytoplasm and nucleus. This result is supported through western analysis of nuclear/cytoplasmic fractions of NkL-TAg cells. Thus, we speculate that the different cellular localization of SERTAD1 reported in different studies reflect the use of different cell types, suggesting that the cellular localization of SERTAD1 is cell-type dependent. Consistent with our speculation, myc-tagged SERTAD1 was also localized in both the cytoplasm and nucleus of NkL-TAg cardiomyocytes (Supplementary Fig. 1). The differential results between the present study and the previous study [29] might also reflect the different types of antibodies used. In the previous study, a mouse monoclonal SERTAD1 antibody was used, whereas in the present study we used a rabbit polyclonal antibody. It is possible that the monoclonal antibody detects only

cytoplasmic SERTAD1, but not nuclear SERTAD1, due to the conformational differences between the two forms of the protein. Further studies are warranted to study the subcellular localization of SERTAD1 in different cell types under different pathophysiological conditions.

Studies using *Sertad1*-inactivated mice showed that this gene is important for maintaining the proper number of pancreatic beta cells [34]. However, no embryonic phenotype was reported. The lack of embryonic heart defects in *Sertad1* knockout mice likely reflects the redundant functions of *Sertad2*, another SERTA gene expressed in embryonic hearts (Fig. 2). *Sertad2* regulates fat storage but is not an essential gene for embryonic development [35]. We speculate that *Sertad1* and *Sertad2* share redundant functions in embryos and further double gene inactivation experiments are required to reveal the embryonic activities of the two genes.

In summary, we identified SERTAD1 as a novel nuclear interaction partner of SMAD1 in mouse embryonic hearts. SERTAD1 acts as a co-activator of SMAD1 to enhance BMP/SMAD-mediated transcription. These results provide crucial information concerning the BMP/SMAD pathway-mediated regulation of cardiogenic gene expression in embryonic hearts.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.127.

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