SMAD4 Is Essential for Human Cardiac Mesodermal Precursor Cell Formation

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Key Words. SMAD4 • Mesoderm • Heart • Human embryonic stem cells

ABSTRACT
Understanding stage-specific molecular mechanisms of human cardiomyocyte (CM) progenitor formation and subsequent differentiation are critical to identify pathways that might lead to congenital cardiovascular defects and malformations. In particular, gene mutations in the transforming growth factor (TGF)β superfamily signaling pathways can cause human congenital heart defects, and murine loss of function studies of a central component in this pathway, Smad4, leads to early embryonic lethality. To define the role of SMAD4 at the earliest stages of human cardiogenesis, we generated SMAD4 mutant human embryonic stem cells (hESCs). Herein, we show that the loss of SMAD4 has no effect on hESC self-renewal, or neuroectoderm formation, but is essential for the formation of cardiac mesoderm, with a subsequent complete loss of CM formation during human ES cell cardiogenesis. Via transcriptional profiling, we show that SMAD4 mutant cell lines fail to generate cardiac mesodermal precursors, clarifying a role of NODAL/SMAD4 signaling in cardiac mesodermal precursor formation via enhancing the expression of primitive streak genes. Since SMAD4 relative pathways have been linked to congenital malformations, it will become of interest to determine whether these may due, in part, to defective cell fate decision during cardiac mesoderm precursor formation.

SIGNIFICANCE STATEMENT
This article defines the role of SMAD4 at the earliest stages of human cardiogenesis. It will also be of considerable interest to scientists interested in human stem cell models of development and disease, physician-scientists interested in congenital heart defects, and basic scientists interested in the transforming growth factor-β signaling pathways.

INTRODUCTION
The transforming growth factor (TGF)β superfamily signaling pathways have been implicated in a diverse array of biological processes, including cell proliferation, differentiation, morphogenesis, tissue homeostasis, and regeneration. The molecular framework for the TGFβ superfamily signaling pathways is well-described [1] with more than 30 ligands, 4 type-II, and 7 type-I receptors. SMAD proteins serve as intracellular mediators and SMAD family member participation in these pathways can be broadly subdivided into two groups: bone morphogenetic protein (BMPs) and TGFβ/Activin/NODAL. BMP signals phosphorylate SMAD1, SMAD5, and SMAD8, while SMAD2 and SMAD3 are phosphorylated in response to activation of the TGFβ, Activin, and NODAL signals. Phosphorylation of the R-SMADs (SMAD1/5/8 and SMAD2/3) permits their association with the common mediator (co-Smad), SMAD4, resulting in nuclear translocation and formation of higher order transcriptional complexes. Thus, SMAD4 plays a central role in TGFβ superfamily signaling.

Both BMP and TGFβ/Activin/NODAL signaling pathways play an important role in human embryonic stem cell (hESC) self-renewal and differentiation. Mouse embryonic stem cells are routinely cultured in medium containing GSK3β and Mek1/2 inhibitors, which can, but not required to, include leukemia inhibitory factor. hESCs use distinct signaling pathways to support their self-renewal. The routine hPSC culture medium contains two essential growth factors: basic fibroblast growth factor (FGF2), TGFβ, or NODAL. FGF2 promotes hESC self-renewal by repressing BMP signaling pathways [2]. TGFβ or NODAL can increase the expression of the pluripotency gene NANOG and leads to consistent long-term culture stability of hESCs [3]. Inhibition of the TGFβ/Activin/ NODAL receptors with SB431542, an inhibitor of Activin A receptor-like kinase ALKS and its relatives ALK4 and ALK7, results in rapid differentiation of hESCs [2, 4–7].
BMP and Activin, on the other hand, can be used to induce differentiation of hPSCs. High-dose Activin A is used to induce mesendodermal differentiation [8], while the combination of BMP and Activin A is used to induce cardiomyocyte (CM) differentiation [9]. Although CM differentiation can be achieved by temporal manipulating WNT signaling pathways [10], Activin A and BMP signaling pathways are still essential. It has been shown that SB431542 can completely block CM differentiation [10]. And BMP inhibitors, such as DMH1, LDN193189 can partially block CM differentiation [11].

Loss-of-function studies in mice have revealed important roles of the TGFβ superfamily signaling pathways in early embryogenesis [12] and later embryonic heart development [13–15]. Previous studies have shown that the loss of function of Smad4 in murine models leads to embryonic lethality before E7.5 with reduced growth and a secondary, noncell autonomous defect in gastrulation [16,17]. Murine Smad4 deficiency results in partial mesoderm formation, with abnormal cardiac development [18,19]. Together, these studies demonstrated that the Smad4-null mice epiblast still maintain the capacity for CM differentiation.

In the human setting, in vitro CM differentiation recapitulates the in vivo regulatory pathways that control the establishment of the corresponding lineage in the early embryo [20,21]. To define whether these studies result in a corresponding effect on human cardiogenesis, we used the CRISPR-Cas9 system to engineer SMAD4 mutated hESCs, allowing the evaluation of the direct cell autonomous role of SMAD4 in both hESC self-renewal and subsequent CM differentiation. First, we demonstrate that SMAD4 is not required for long-term maintenance of hESCs. Second, we show that SMAD4 is essential for cardiac mesodermal precursor cell formation, and reveal the distinct roles of WNT/β-catenin and NODAL/SMAD4 signaling pathways at the earliest step of human cardiogenesis.

**Materials and Methods**

**Maintenance of hESCs**

Human pluripotent stem cells (ES03, NIH code: HES3) were obtained from WiCell and maintained on Vitronectin (Thermo Fisher Scientific, USA) coated plates in Essential 8 (Thermo Fisher Scientific). And Versene (Thermo Fisher Scientific) was used to dissociate the hESCs for passage.

**Cardiac Differentiation of hESCs Via the GIWI Method**

After the hESCs achieved confluence on a Vitronectin-coated surface, the cells were dissociated with Versene (Thermo Fisher Scientific) at 37°C for 5 minutes and then seeded onto a Matrigel-coated cell culture dish at 100,000 cells per cm² in Essential 8 supplemented with 5 μM Y-27632 (Sigma–Aldrich; day –2) for 24 hours. Cells were then cultured in Essential 8 without Y-27632, and changed daily. Cardiac differentiation of hESCs was performed according to previously published growth factor based methods [10,22–24]. Briefly, at day 0, cells were treated with 100 ng/ml Activin A (R&D Systems Inc., Minneapolis, MN) in RPMI/B27-insulin. After 24 hours, the medium was changed to RPMI/B27-insulin supplemented with 5 ng/ml BMP4 (R&D Systems Inc.) for the next 4 days. At day 5, the medium was changed to RPMI/B27 and medium was changed every 2 or 3 days.

**Plasmid Constructs**

CRISPR-Cas9. PiggyBac CRISPR-Cas9 (PB-CRISPR) was constructed by cloning the sgRNA and Cas9 expression cascade from pLentiCRISPR [25] into a PiggyBac vector modified from PB-OSKML [26]. We designed two gRNAs targeting exon 3 of SMAD4, smad4-g1: TGATCTATGCCCGTCTCTGG and smad4-g2: AGACGGGCATAGATCACATG. The two gRNAs were cloned into PB-CRISPR similar to pLentiCRISPR vector, termed pPB-CRISPR-SMAD4-g1 and pPB-CRISPR-SMAD4-g2.

PiggyBac Ubc-Smad4V5. V5-tagged SMAD4 cDNA and GFP–Blasticidin chimeras were synthesized using IDT’s Gene Block, and the fragment’s cloned into modified PB-Ubc vector. P2A sequence linked the SMAD4V5 with a chimera protein GFPBsd, which functions as both GFP and Blasticidin resistance. The whole cascades were integrated into the genome using PiggyBac System [26].

**Cell Line Generation**

**SMAD4 Mutant hESC Lines.** Three plasmids, pPB-CRISPR-SMAD4-g1, pPB-CRISPR-SMAD4-g2 and pCyL43, were used for genome editing in hESCs. Two million hESCs, 1 μg pCyL43 and 4 μg pPB-CRISPR-SMAD4-g1, pPB-CRISPR-SMAD4-g2 DNA were mixed in 100 μl nucleofection solution and then nucleofected with program B-16 using a Nucleofector 2b device (Lonza, Germany). After 2 weeks of 0.5 μg/ml Puromycin selection, cells were singularized and sparsely seeded to generate single cell derived clones.

**SMAD4 Re-Expression hESC Lines.** Two plasmids, pPB-Ubc-SMAD4V5 and pCyL43, were used to generate SMAD4 overexpression/rescue hESCs. Two million hESCs, 1 μg pCyL43 and 4 μg pPB-Ubc-SMAD4V5 DNA were mixed in 100 μl nucleofection solution and then nucleofected with program B-16 using a Nucleofector 2b device (Lonza). After 2 weeks of 5 μg/ml Blasticidin selection, cells were singularized and sparsely seeded to generate single cell derived clones. Colonies with homogeneous GFP expression were picked for final rescue experiments.
SMAD4 and Human Cardiac Mesodermal Progenitors

RESULTS

SMAD4 Is Not Required for hESC Self-Renewal

hESCs can be cultured long-term with the maintenance of pluripotency using a chemically defined E8 medium, but in the absence of TGFβ or NODAL, hESCs can become unstable and will differentiate after long-term passage [3]. Since SMAD4 is an irreplaceable component in these signaling pathways, SMAD4 mutant hESCs might interrupt TGFβ/NODAL signaling, leading to instability following long-term passage. Despite the clear requirement of TGFβ/NODAL signaling in the maintenance of pluripotency, the requirement of SMAD4 is still unclear.

Accordingly, we used the CRISPR-Cas9 system to generate six SMAD4 mutated hESC clonal cell lines, with further analysis of two independent lines. The targeted region was sequenced, confirming that the two alleles of clone 1 and 2 had deletions in the targeted site (Fig. 1A). At the protein level, both Western blot and immunostaining confirmed the SMAD4 mutant hESCs cannot produce stable SMAD4 proteins (Fig. 1B, 1C). The success of generating SMAD4 mutant hESC clones demonstrated their self-renewing capacity. SMAD4 mutants can be expanded and passed in E8 medium without morphological defects in standard culture up to 6 months (Fig. 1B). Furthermore, all of the SMAD4 mutant clones expressed the pluripotency markers: POU5F1, NANOG, and SOX2 (Fig. 1C and Supporting Information Fig. S4A).

The E8 medium we used, was based on FGF2 and TGFβ. Since FGF2 and NODAL can also support pluripotency, we further tested the alternative medium. We added 100 ng/ml FGF2 to E6, which is E8 without FGF2 and TGFβ, as a basal medium, which we designated as medium E7. In total, four media were tested, E7, E7 with 100 ng/ml NODAL, E7 with 2 ng/ml TGFβ, and E7 with 2 ng/ml Activin A. Since the SMAD4 mutants cannot respond to NODAL, TGFβ, or Activin A, they can be maintained in all four media, while wild-type E03 differentiated in E7 and Activin A after a few days in culture (Supporting Information Fig. S3). This result further demonstrates that SMAD4 is not required for hESC self-renewal.

We also used RNA-Seq to compare wild-type and SMAD4 mutant hESCs. In SMAD4 mutants, splicing appeared normal (Supporting Information Fig. S1A, S1B), but the DNA-binding domain was disrupted (Supporting Information Fig. S1C).

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Mutation in one allele of clone 2 introduced a premature stop codon, whereas the two alleles in clone 1 and the other allele in clone 2 might produce mutated proteins. All mutations were found in the link between the β-hairpin and the L4 double loop within the DNA-binding domain of the MH1 domain [30].

Differential gene expression analysis of the wild-type and SMAD4 mutant cell lines revealed minimal differences (Supporting Information Fig. S1D and Table S1). Pluripotency genes, including POU5F1, NANOG, SOX2, were not differentially expressed (Supporting Information Table S1). In contrast,
TGFβ signaling and target genes including LEFTY1, LEFTY2, and CER1 (Supporting Information Table S1), were down regulated as expected, supporting SMAD4 loss-of-function. Taken together, these data demonstrate that SMAD4 is not essential for hESC self-renewal.

**SMAD4 Is Essential for CM Differentiation**

In early differentiation protocols, BMP4 and Activin A were used to differentiate hESCs to cardiomyocytes [9]. Both BMP4 and Activin A require SMAD4 for proper function. Although some BMPs or Activin A inhibitors have been shown to block or reduce the differentiation process [10, 11], direct evidence is lacking to show that SMAD4 is essential for cardiac mesodermal formation and/or downstream CM differentiation.

To determine if SMAD4 is essential for human CM differentiation, we compared wild type (WT) and SMAD4 mutant hESCs in a CM differentiation assay using the GiWi protocol [10]. Though WT differentiated hESCs produced robust beating CMs, after 14 days, SMAD4 mutants produced no mature beating cells ($n = 4$). Furthermore, we tested both WT and mutant SMAD4 hESC GiWi treated cells at day 14 for the expression of TNNT2, a marker of CM sarcomere formation, via flow cytometry. Similar to the functional beating CM assay, more than 80% of WT hESCs showed expression of TNNT2, while both SMAD4 mutant clones produced no detectable TNNT2+ cells (Fig. 2A and Supporting Information Fig. S3). To our knowledge this result is the first direct evidence that SMAD4 is absolutely essential for human CM differentiation from hESCs.

To prove that the failure of CM differentiation is due to the loss of SMAD4, we re-expressed SMAD4 in the mutant cells using a human Ubiquitin C promoter (Ubc) driving the expression of a C-terminal V5 tagged SMAD4. We examined transfected hESC cells by immunostaining of POU5F1, SOX2, and V5, marking expression of tagged SMAD4. SMAD4 mutants stained positive for POU5F1, SOX2, but not V5, while the rescued mutants stained positive for V5, indicating the expression of the tagged SMAD4 (Supporting Information Fig. S4A). Following differentiation using the GiWi protocol, both rescued clones had only a few beating clusters and the flow cytometry showed 1% of TNNT2+ cells (Supporting Information Fig. S5). This is in contrast to the nonrescued mutant clones that produced no detectable CMs (Fig. 2C, 2D and Supporting Information Fig. S6). These data strongly support the notion that in the absence of SMAD4, hESCs cannot differentiate into CM, but the level and duration was regulated by SMAD4. Consistent with a previous study [32], we noted the presence of PS genes in SMAD4 mutant cells was clearly weaker than wild-type hESC (Fig. 2D and Supporting Information Fig. S7B, S7C), suggesting these might be the downstream targets of SMAD4. It was notable that NODAL1 was one of the very few TGFβ pathway ligands expressed (Fig. 3C). Furthermore, NODAL was expressed in both wild-type and SMAD4 mutants, suggesting its expression was induced through WNT/β-catenin signaling. This result supports the notion that in the absence of SMAD4, hESCs cannot respond to NODAL, NODAL/SWIM4 downstream targets remain inactive, and fail to induce the molecular program necessary for the earliest stages of cardiogenesis.

To further study the initial formation of cardiac mesoderm, we examined the effects of WNT/β-catenin activation at day 1. As the first step of GiWi protocol, high-dose GSK3β inhibitor (CH99021, 12 μM) activated the primitive streak (PS) genes: T (TBXT), EOMES, MIXL1, and GSC (Fig. 3D). The expression of PS genes in SMAD4 mutant cells was clearly weaker than wild-type cells (Fig. 3D and Supporting Information Fig. S7B, S7C), suggesting the initial expression of PS genes might be dependent on WNT/β-catenin, but the level and duration was regulated by NODAL/SWIM4. Consistent with a previous study [32], we noted the proneuroectodermal genes: Gbx2, Axin2 were also induced (Fig. 3D). These results untangle the roles of WNT/β-catenin and NODAL/SWIM4 signaling pathways in human mesoderm induction. With WNT stimulation, primitive streak (PS) genes are expressed, but relatively weakly, and it appears that the NODAL/SWIM4 pathway enhances PS gene expression and takes the activated cells into formation of mesodermal lineages.

**RNA-Seq Analysis Reveals the Distinct Roles of WNT/β-Catenin and NODAL/SWIM4 Signaling Pathways**

As established in a previous study in hESCs [31], in vitro cardiogenesis occurs via sequential distinct developmental stages, corresponding to the parental embryonic stem cells (day 0), primitive streak/early mesoderm (day 1), cardiac mesoderm (day 3), and cardiac progenitor/early CM stages (day 6). To understand the downstream effect of SMAD4 mutations in this sequential process of human in vitro cardiogenesis, we used RNA-Seq to comprehensively compare the transcriptional profiles between the WT and the SMAD4 mutants at these stages. The relationship between the SMAD4 mutant and the WT cell lines is displayed in the multidimension scaling plot (Fig. 3A). During differentiation, the number of differentially expressed genes increased beginning with hESCs, 12 up, 8 down and by day 6 of differentiation, 223 up, 573 down (log fold change, log; FC > 3, $p$-value <10−5; Fig. 3B).

We subsequently compared the expression of ligands from three major signaling pathways (TGFβ superfamily, WNT, and FGF) between SMAD4 mutants and wild-type differentiating hESC (Fig. 3C). At day 1 after induction, the signaling cues for initiating the differentiation program were formed in WT cells, including DKK4, DKK1, FGF4, FGF19, FGF13, FST, LEFTY1, NODAL, and WNT5B. In contrast, SMAD4 mutants failed to induce DKK1, DKK4, FGF4, and FGF13, suggesting these might be the downstream targets of SMAD4. It was notable that NODAL was one of the very few TGFβ pathway ligands expressed (Fig. 3C). Furthermore, NODAL was expressed in both wild-type and SMAD4 mutants, suggesting its expression was induced through WNT/β-catenin signaling. This result supports the notion that in the absence of SMAD4, hESCs cannot respond to NODAL, NODAL/SWIM4 downstream targets remain inactive, and fail to induce the molecular program necessary for the earliest stages of cardiogenesis.

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**SMAD4 Is Required for the Formation of Human Cardiac Mesodermal Precursor Cells**

Consistent with reduced expression of PS genes, there was a failure of mesendoderm induction, attenuating the expression of CXCR4, HAND1, MESP1, SOX17, and TBX6 (Fig. 3D and Supporting Information Fig. S7A). However, at the protein level, mesoderm markers, such as APLNR, ANPEP (CD13), CXCR4,
were expressed in day 2 cells (Supporting Information Fig. S8). This suggests that these genes were stimulated by WNT/β-catenin, but required SMAD4 relative signaling pathways to correctly direct cells into the mesendoderm cell fate. We also used immunostaining to verify that the SMAD4 mutants failed to form MESP1+ cardiac mesodermal precursor cells. Compared with the wild-type cells, the expression of MESP1 in the SMAD4 mutants was much lower. Although the cells were still in the early stage, cardiac progenitor marker ISL1 and early CM marker NKX2-5 also were expressed at a very low level in

**Figure 2.** SMAD4 is required for human cardiomyocyte differentiation. (A): Flow cytometry analysis demonstrates SMAD4 mutant human embryonic stem cells (hESCs) fail to form TNNT2+ cardiomyocytes. Compare left panel hESC with middle and right panel mutant SMAD4 clones C1 and C2, respectively. (B): SMAD4 rescue construct used to overexpress/rescue SMAD4 in wild-type and the mutant clones contains a Ubc promoter driving V5-tagged SMAD4. (C): Flow cytometry analysis demonstrates rescued clones rescue cardiac differentiation to became TNNT2+ cardiomyocytes. (D): Flow cytometry analysis demonstrates SMAD4 mutated hESCs failed to become TNNT2+ cardiomyocyte using growth factor-based differentiation method. (E): Summary graph of TNNT2 flow cytometry of SMAD4 WT, mutant clones, rescue clones, and growth factor-based differentiation method. *, p < .0001; compared with ES03 (n ≥ 3). #, p < .0001; compared with SMAD4.C1 (n = 4). †, p < .0001; compared with SMAD4.C2 (n = 4). ES03 + Rescue.C2 with similar level SMAD4v5 expression as the mutant rescue clones. Ns, p = .2664 (n = 4). ‡, p < .0001; compared with ES03.AB (n = 3). AB indicates Activin a/BMP4 differentiation protocol.
the wild-type. However, in the SMAD4 mutants, ISL1 and NKX2-5 were at negligible levels below the level of detection. Taken together, these data suggest the SMAD4 mutants fail to form cardiac mesoderm precursor cells.

By day 6, wild-type differentiating hESCs became ISL1+ cardiac progenitors [33] and began to express early CM markers: NKX2-5, TNNT2, MYL7, and PLN. At the same stage of differentiation, SMAD4 mutant cells did not express any of these cardiac genes. In contrast, SMAD4 mutant cells expressed FOXG1, PAX6, RAX, and SIX6, consistent with a neuroectoderm fate [34] (Fig. 3D). We also confirmed the expression of PAX6 in the mutant cells, but not in wild-type, by immunostaining (Fig. 4B). Taken together, these results demonstrate that the NODAL/SMAD4 pathway plays an important role in the early cell fate decision between mesendoderm and neuroectoderm in human cells, and it essential for the formation of cardiac mesoderm precursor cells.

**DISCUSSION**

**SMAD4 and hESC Self-Renewal**

Early studies have demonstrated the essential roles for TGFβ and NODAL signaling play in hESC self-renewal [2, 4–6]. SMAD4 plays a critical role in the modulation of TGFβ signaling, suggesting it would be important for hESC self-renewal. Avery et al. first challenged this idea showing that SMAD4 knockdown in hESCs prevents the rapid differentiation normally induced by the TGFβ inhibitor SB431542. Furthermore, Avery observed a loss of the stability of hESC cultures and differentiation toward neural lineage. Thus, their result demonstrated that SMAD4 was not required for hESC self-renewal in the short-term. Our results agree with this short-term assessment. However, while Avery’s SMAD4 KD cells were unstable in the long-term, our SMAD4 mutant cells are stable in the

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long-term. There are several possible explanations for these differences. First are differences in culture conditions—we used a chemically defined media, Essential 8, whereas, Avery used a Dulbecco’s modified Eagle’s medium supplemented with knock-out serum replacement, nonessential amino acids, L-glutamine, and βIFGF. Another possible explanation for the discrepancy between Avery’s work and ours is the techniques used to eliminate SMAD4 expression. Avery used a knockdown approach that may have contained residual, functional SMAD4. In contrast, we inactivated SMAD4 using genetic techniques. Our SMAD4 mutants demonstrate a loss of both SMAD4 protein and SMAD4 function. Thus, in the current study, we demonstrate SMAD4 is dispensable for hESC long-term self-renewal.

**SMAD4 and Human Cardiac Mesodermal Formation and CM Differentiation**

One of the central advantages of human ES cell models of cardiogenesis lies in the ability to study the role of specific signaling pathways at the earliest stages of mesodermal formation. Herein, we document the sequential expression of primitive streak marker TBXT at day 1, cardiac mesoderm marker MESP1 at day 3, and cardiac progenitor marker, ISL1, at day 6.

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**Figure 4.** SMAD4 mutant fail to form cardiac mesoderm precursor cells and defaults to neuroectoderm fate under conditions for cardiac differentiation. **(A):** Immunostaining demonstrates expression of the cardiac progenitor marker ISL1, cardiac mesoderm marker MESP1 and cardiomyocyte marker NKX2-5 after 3 days cardiac differentiation. Scale bar = 100 μm. **(B):** Immunostaining demonstrates expression of the pluripotent marker POU5F1, cardiac marker NKX2-5, and neuroectoderm marker PAX6 after 6 days cardiac differentiation. Scale bar = 100 μm.
However, in SMAD4 mutants, TBXT was expressed at a lower level than in wild-type hESCs, while the expression of MESP1 and ISL1 were absent in SMAD4 mutants. Mechanistically, our RNA-Seq results suggest that the differentiation programs diverge at the PS stage, with lower expression level of PS genes, including TBXT, EOMES, and MIXL1. This suggests that the trigger of these genes was present, but the right positive feedback or the necessary enhancer binding was missing. Previous studies [32, 35] showed that β-catenin can trigger the expression of PS genes. But without the feedback from NODAL/SMAD4 signaling pathway, these genes could not reach the levels that are required to drive the downstream signaling cascades. When the first wave of signal cubes fades away, the cells just drop back to their default neuroectoderm fate (Fig. 5). Thus, the enhancement of PS gene expression from NODAL/SMAD4 signaling pathway is essential for human cardiac mesodermal formation and subsequence CM differentiation.

Distinct Role of SMAD4 in Human Versus Murine Models of Cardiogenesis

Homozygous Smad4 null mice exhibit growth retardation, develop abnormal visceral endoderm, and fail to form mesoderm [16, 17]. Aggregation of mutant Smad4 mouse embryonic stem cells (mESCs) with wild-type tetraploid morulae rescues the gastrulation defect [17], suggesting that the failure to gastrulate and form mesoderm is secondary and noncell autonomous. Inactivation of Smad4 at the epiblast stage with Sox2Cre permits gastrulation and mesoderm formation. The expression of cardiac differentiation stage markers Tbx1, Mesp1, Nkx2-5, and Actc1 can be detected at corresponding stages [18, 19]. In the absence of Smad4, the cardiac development of the heart is severely compromised with multiple abnormal structures, though in some mutant embryos, a primitive, and unlooped heart tube is observed [19]. This indicates that in mice, cardiac lineage specification occurs in the absence of Smad4. In contrast, our current study demonstrates that human SMAD4 mutants fail to form either cardiac mesoderm or cardiomyocytes, reflecting the differential requirements of SMAD4 in early cardiogenesis between human and mice.

CONCLUSION

Existing human genetic data supports our in vitro findings. In humans, recessive SMAD4 somatic mutations are strongly correlated with the development of pancreatic and other cancers [36, 37]. Germline haploinsufficiency of SMAD4 is associated with the autosomal dominant syndrome juvenile polyposis/hereditary hemorrhagic telangiectasia [38, 39]. In contrast, heterozygous gain of function SMAD4 mutations are associated with Myhre Syndrome that includes cardiac structural defects [40]. Furthermore, a subtle SMAD4 mutation (rs281875322) was reported in a patient with aortic arch hypoplasia and a ventricular septal defect [41]. To date, 539 SMAD4 variants have been reported in ClinVar [42], yet no homozygous mutations have been reported in humans. The lack of any existing report of a human with homozygous SMAD4 mutations supporting SMAD4’s essential role in human development. The current studies suggest a close examination of other known or novel genetic variants for congenital cardiac malformations may also be found at the earliest stages of cardiac mesodermal formation in human pluripotent stem cell model systems.

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AUTHOR CONTRIBUTIONS

J.X.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; P.J.G.: data analysis and interpretation, manuscript writing; K.R.C.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
REFERENCES

1 Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol 2012;13:616–630.
2 Xu RH, Peck RM, Li DS et al. Basic FGF and suppression of BMP signalling sustain undifferentiated proliferation of human ES cells. Nat Methods 2005;2:185–190.
3 Chen G, Gulbranson DR, Hou Z et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8:424–429.
4 Xu RH, Sampsel-Barron TL, Gu F et al. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell 2008;3:196–206.
5 James D, Levine AJ, Besser D et al. TGFbeta/activin/nodal signalling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 2005;132:1273–1282.
6 Vallier L, Mendjan S, Brown S et al. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development 2009;136:1339–1349.
7 Avery S, Zafarana G, Gokhale PJ et al. The role of SMAD4 in human embryonic stem cell self-renewal and stem cell fate. Stem Cells 2010;28:863–873.
8 D’Amour KA, Agulnick AD, Eliazer S et al. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 2005;23:1534–1541.
9 Kattman SJ, Witty AD, Gagliardi M et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 2011;8:228–240.
10 Lian X, Hsiao C, Wilson G et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci USA 2012;109:E1848–E1857.
11 Burridge PW, Matza E, Shukla P et al. Chemically defined generation of human cardiomyocytes. Nat Methods 2014;11:855–860.
12 Goumans MJ, Mummery C. Functional analysis of the TGFbeta receptor/Mad/Smad pathway through gene ablation in mice. Int J Dev Biol 2000;44:253–265.
13 Wang J, Xu N, Feng X et al. Targeted disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure. Circ Res 2005;97:821–828.
14 Qi J, Yang G, Yang L et al. Essential role of Smad4 in maintaining cardiomyocyte proliferation during murine embryonic heart development. Dev Biol 2007;311:136–146.
15 Song L, Yan W, Chen X et al. Myocardial smad4 is essential for cardiogenesis in mouse embryos. Circ Res 2007;101:277–285.
16 Yang X, Li C, Xu X et al. The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proc Natl Acad Sci USA 1998;95:3667–3672.
17 Sirard C, de la Pampa JL, Elia A et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev 1998;12:107–119.
18 Costello I, Pimeis IM, Drager S et al. The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation. Nat Cell Biol 2011;13:1084–1091.
19 Chu GC, Dunn NR, Anderson DC et al. Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. Development 2004;131:3501–3512.
20 Murre CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. Cell 2008;132:661–680.
21 Spater D, Hansson EM, Zangi L et al. How to make a cardiomyocyte. Development 2014;141:4418–4431.
22 Zhang J, Klos M, Wilson GF et al. Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: The matrix sandwich method. Circ Res 2011;112:1125–1136.
23 Birkef MJ, Casini S, Kosmidis G et al. PGC-1alpha and reactive oxygen species regulate human embryonic stem cell-derived cardiomyocyte function. Stem Cell Res 2013;13:560–574.
24 Laflamme MA, Chen KY, Naumova AV et al. Cardiomyocytes derived from human embryonic stem cells in prosurvival factors enhance function of injured rat hearts. Nat Biotechnol 2007;25:1015–1024.
25 Shalem O, Sanjana NE, Hartenstein E et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 2014;343:84–87.
26 Yusa K, Rad R, Takeda J et al. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat Methods 2009;6:363–369.
27 Dobin A, Davis CA, Schlesinger F et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
28 Liao Y, Smyth GK, Shi W. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2014;30:923–930.
29 Robinson MD, McCarthy DJ, Smyth GK. edgeR: A bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139–140.
30 Jones JB, Kern SE. Functional mapping of the NH1 DNA-binding domain of DPC4/SMAD4. Nucl Acids Res 2000;28:2363–2368.
31 Foo KS, Lehtinen ML, Leung CY et al. Human ISL1(+) ventricular progenitors self-assemble into an in vivo functional heart patch and preserve cardiac function post infarction. Mol Ther 2018;26:1644–1659.
32 Yuna NS, Schachter KA, Lerdrup M et al. Beta-catenin regulates primitive streak induction through collaborative interactions with SMAD2/SMAD3 and OCT4. Cell Stem Cell 2015;16:639–652.
33 Bu L, Jiang X, Martin-Puig S et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 2009;460:113–117.
34 Meyer JS, Shearer RL, Capowski EE et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci USA 2009;106:16698–16703.
35 Lian X, Xu J, Bao X et al. Interrogating canonical Wnt signaling pathway in human pluripotent stem cell fate decisions using CRISPR-Cas9. Cell Mol Bioeng 2016;9:325–334.
36 Hahn SA, Hoque AT, Moskaluk CA et al. Homozygous deletion map at 18q21.1 in pancreatic cancer. Cancer Res 1996;56:490–494.
37 Schutte M, Hruban RH, Hedrick L et al. DPC4 gene in various tumor types. Cancer Res 1996;56:2527–2530.
38 Gallione CJ, Repetto GM, Legius E et al. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet 2004;363:852–859.
39 Howe JR, Roth S, Ringold JC et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 1998;280:1086–1088.
40 Myhre SA, Ruvalcaba RH, Graham CB. A new growth deficiency syndrome. Clin Genet 1981;20:1–5.
41 Jin SC, Homsy J, Zaidi S et al. Contribution of rare inherited and de novo variants in 2,871 congenital heart disease probands. Nat Genet 2017;49:1593–1601.
42 Landrum MJ, Lee JM, Benson M et al. ClinVar: Public archive of interpretations of clinically relevant variants. Nucl Acids Res 2016;44:D862–D868.