# Mesp1 Acts as a Master Regulator of Multipotent Cardiovascular Progenitor Specification

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### **SUMMARY**

During embryonic development, multipotent cardiovascular progenitor cells are specified from early mesoderm. Using mouse ESCs in which gene expression can be temporally regulated, we have found that transient expression of Mesp1 dramatically accelerates and enhances multipotent cardiovascular progenitor specification through an intrinsic and cell autonomous mechanism. Genome-wide transcriptional analysis indicates that Mesp1 rapidly activates and represses a discrete set of genes, and chromatin immunoprecipitation shows that Mesp1 directly binds to regulatory DNA sequences located in the promoter of many key genes in the core cardiac transcriptional machinery, resulting in their rapid upregulation. Mesp1 also directly represses the expression of key genes regulating other early mesoderm and endoderm cell fates. Our results demonstrate that Mesp1 acts as a key regulatory switch during cardiovascular specification, residing at the top of the hierarchy of the gene network responsible for cardiovascular cell-fate determination.

### INTRODUCTION

The heart is the first functional organ that is formed during embryonic development. The ancestor of the primitive heart is estimated to have arisen about 500 million years ago, since the core transcriptional machinery required for cardiac differentiation has been extremely conserved during evolution (Davidson and Erwin, 2006; Olson, 2006; Satou and Satoh, 2006). Cardiac progenitors are specified during the early stages of gastrulation as soon as the epiblast cells are incorporated within the cranial portion of the primitive streak. Around embryonic day 6 (E6.0) in mice, the prospective cardiogenic mesoderm migrates away from the primitive streak bilaterally and cranially to form the anterior and lateral plate mesoderm (E6.5), where the cardiac crescent, corresponding to the primary heart field, appears at around E7.0 (Abu-Issa and Kirby, 2007; Buckingham et al., 2005; Garry and Olson, 2006). The cells of the cardiac crescent migrate medially and form a linear single heart tube, which will give rise to the cells of the left ventricle and atria. Complex looping and rotation of the heart tube, as well as the addition of cells coming from the secondary heart field, giving rise to the right ventricle and the outflow track, generate a four-chambered heart (Buckingham et al., 2005; Laugwitz et al., 2008).

Various lines of evidence have suggested that different cardiac cell types, including cardiomyocytes, endothelial cells, smooth muscle cells, as well as conduction cells, which compose the mature heart tissue, arise from the differentiation of multipotent cardiovascular progenitors (MCPs) generated soon after gastrulation (Garry and Olson, 2006; Moretti et al., 2006; Wu et al., 2006). Recent studies also provide compelling evidence that different sources of MCPs, which are specified during embryonic development, are also generated during pluripotent embryonic stem cell (ESC) differentiation (Murry and Keller, 2008). These MCPs can be isolated on the basis of Brachyury and Flk1 expression for early cardiac progenitors (Kattman et al., 2006; Kouskoff et al., 2005; Yang et al., 2008) and using Nkx2-5 and Islet1 reporter genes for isolating cardiovascular progenitors of the primary (Nkx2-5) and the secondary heart fields (Nkx2-5 and Islet1) (Moretti et al., 2006; Wu et al., 2006). ESC differentiation represents a very attractive model to dissect the molecular mechanisms implicated in cardiovascular cell-fate specification but also may represent an ideal source of cardiovascular cells for cellular therapy in humans (Martin-Puig et al., 2008).

Although the gene regulatory network of transcription factors involved in cardiac determination, proliferation, patterning, and differentiation is relatively well known, the early molecular mechanism that induces the specification of multipotent cardiovascular cell fate from undifferentiated mesoderm remains largely unknown (Abu-Issa and Kirby, 2007; Olson, 2006). It is still unclear what are the intrinsic or extrinsic factors that induce cardiovascular progenitor specification and coordinate the expression of cardiac transcription factors at the right time and at the right place.

Expression of Mesp1, a transcription factor of the b-HLH family, is one of the earliest signs of cardiovascular development in vertebrates (Saga et al., 2000). During gastrulation in mice, Mesp1 is strongly expressed at the onset of gastrulation (E6.5) along the primitive streak and in the prospective cardiac mesoderm and is then rapidly downregulated after E7.5 (Saga et al., 1996). Lineage-tracing experiments using mice in which the Cre recombinase has been knocked in into the *Mesp1* locus demonstrated that most cardiac cells and some vascular cells arise from cells that expressed *Mesp1* at one point of their development (Saga et al., 1999). *Mesp1* null mice present a severe defect in heart morphogenesis that has been attributed to a delay in



cardiac progenitor migration (Saga et al., 1999). The double *Mesp1/2* null mice exhibit a major defect in gastrulation resulting in the absence of mesoderm formation (Kitajima et al., 2000). This dramatic and early gastrulation defect has precluded molecular characterization of the specific role of Mesp1/2 during cardiac specification, and left open the question about the precise mechanism by which Mesp1 acts during cardiac development. Does Mesp1 only promote the migration of cardiac progenitors toward the lateral plate mesoderm, or does Mesp1 instruct the primitive mesoderm to become MCPs?

To dissect the cellular and the molecular mechanism by which Mesp1 acts during cardiac specification, we took advantage of the natural differentiation of ESCs into MCPs and the ease of their genetic manipulation (Murry and Keller, 2008). We showed that, during ESC differentiation, Mesp1 is expressed very transiently, reminiscent of its expression during embryonic development. By transiently overexpressing Mesp1 during ESC differentiation, we drastically accelerated and promoted specifically cardiovascular cell fate through a cell autonomous mechanism. Using genome wide transcriptional analysis after Mesp1 induction, we uncovered a discrete set of genes that are rapidly regulated upon Mesp1 induction. We showed that Mesp1 both directly activated many key genes belonging to the core cardiac transcriptional machinery and directly repressed genes promoting early mesoderm and endoderm cell-fate specification. Mesp1 first transiently stimulated its own endogenous expression through a direct positive autoregulatory loop and then inhibited its own expression, therefore, acting as a molecular switch during cardiac specification. Altogether, our results provide compelling evidence that Mesp1 acts as a cardiovascular master regulator during specification of MCPs during ESC differentiation.

## RESULTS

## Transient Expression of Mesp1 Dramatically Accelerates and Increases Cardiac Differentiation from ESCs

We first examined by RT-PCR the temporal expression of key transcription factors implicated in the transition from pluripotent ESCs to cardiac terminal differentiation (Figure 1A). As previously shown (Kattman et al., 2006; Kouskoff et al., 2005; Liu et al., 2007), when pluripotent ESCs are induced to differentiate, the temporal appearance of the key transcriptional factors implicated in mesoderm and cardiac commitment is very similar to the temporal expression of these genes during embryonic devel-

opment (Murry and Keller, 2008). Genes regulating the specification of the primitive streak, such as *Brachyury*, are strongly and rapidly upregulated. *Mesp1* began to be expressed soon after, peaked at day 4, and then was rapidly downregulated. Key cardiac transcription factors began to be expressed at days 3 to 4, peaking around day 6. Cardiac structural genes, such as *troponinT*, began to be expressed at day 5 and peaked at day 8, and their expression was maintained thereafter, in good accordance with the contractile phenotype of the cells observed upon microscopic inspection (Figure 1B).

To study the role of Mesp1 during cardiac cell-fate specification, we generated a recombinant ESC line in which the expression of an epitope-tagged version of Mesp1 followed by an IRES-GFP can be temporally and specifically induced upon doxyclin (Dox) addition (Figure S1A available online) (Kyba et al., 2002). To determine whether Mesp1 directly promotes cardiac specification, we induced the expression of Mesp1 from day 2 to day 3, 1 day earlier than its endogenous expression, and monitored the temporal appearance of beating EBs. In the absence of Dox, no expression of transgene was detectable by FACS, western blot, or immunostaining analysis (Figures S1B-S1D). Upon Dox addition, Mesp1 is rapidly induced, and 12 hr after Dox administration, Mesp1 is clearly seen in the nucleus of ESCs. After 24 hr, about 80% of ESCs expressed Mesp1 (Figures S1B-S1D). The precocious expression of Mesp1 resulted in an acceleration of cardiac differentiation as demonstrated by the premature appearance of beating cells in the EB culture, which occurred at day 7, a day earlier than in untreated cells or control GFP-inducible ESCs treated with Dox (Figure 1B). A close observation of the EBs revealed an increased number of beating zones within EBs that have been stimulated with Mesp1 (Movies S1 and S2). Immunofluorescence and FACS analysis demonstrated the precocious and increased expression of TroponinT, a cardiac specific marker, following Mesp1 induction (Figures 1C-1F). Mesp1-stimulated cells generated four to five times more cardiac cells (Figures 1E and 1F), which represents one, if not the greatest, promotion of cardiac differentiation induced by a single factor.

## Mesp1 Specifically Promotes the Specification of Multipotent Cardiovascular Progenitors from Primitive Mesoderm

During embryonic development or ESC differentiation, cardiac cells are thought to arise from the differentiation of MCPs (Murry and Keller, 2008). To determine whether Mesp1 promotes the specification of MCPs or whether its effect is restricted to the

Figure 1. Precocious Expression of Mesp1 Dramatically Accelerates and Increases Cardiac Differentiation from ESCs

(A) Expression profiles of early mesodermal (*Brachyury* and *Mesp1*) and cardiac transcripts (*Nkx2-5*, *Gata4*, *Mef2c*, *Hand2*, and *TroponinT2*) during normal ESC differentiation as measured by RT-PCR. Data represent relative expression of transcripts compared to undifferentiated ESCs as mean ± standard error of the mean (SEM) of three biologically independent experiments.

<sup>(</sup>B) A pulse of Mesp1 (day 2 [D2] and day 3 [D3]), before its endogenous expression, accelerates cardiac commitment in EBs as detected by the precocious appearance of beating areas, compared to unstimulated or GFP overexpressing cells, while a continuous administration of Dox inhibits cardiac differentiation. Data represent mean ± SEM of three biologically independent experiments.

<sup>(</sup>C) EBs were stained with anti-troponinT antibody, a specific marker of cardiomyocytes. Mesp1 expression during D2 and D3 induced precocious troponinT expression. Squares indicate areas of magnification for each condition shown on the right of the panel.

<sup>(</sup>D and E) Quantification of troponinT expression during ESC differentiation by FACS. At different times, EBs were dissociated, stained for troponinT and analyzed by FACS. Data represent mean ± SEM of three biologically independent experiments.

<sup>(</sup>F) Absolute enrichment in troponinT positive cells following transient Mesp1 expression. The percentage of troponinT-positive cells was adjusted for the total number of cells presented in both conditions (see Figure S4).



promotion of cardiac differentiation, we analyzed, by RT-PCR and immunostaining, the expression of markers specific for different types of mature cardiovascular cells. Mesp1 increased the expression of cardiac transcription factors (Figure 2A), pancardiac markers (Figures 1C and 2A; Figure S2A), ventricular markers (Figures 2A and 2B), atrial markers (Figures 2A and 2C), as well as markers of pace maker cells (Figure 2A). In addition to promoting myocardial differentiation, Mesp1 also accelerated and promoted the differentiation of vascular cells as shown by the precocious and increased expression of CD31 and VE-Cadherin (Figures 2A and 2D) and smooth muscle cells as shown by expression of smooth muscle actin (SMA) (Figure 2E). Altogether, the three main cell lineages arising from the differentiation of MCPs represent about 50%-60% of all cell lineages following Mesp1 induction. The similar promotion and acceleration of ESC differentiation into cardiac, vascular, and smooth muscle cells suggested that Mesp1 induced the specification of MCPs rather than just promoting cardiac differentiation.

Two distinct sources of cardiogenic mesoderm give rise to MCPs during embryonic development and ESC differentiation (Buckingham et al., 2005; Laugwitz et al., 2008). The primary heart field originates from the anterior splanchnic mesoderm and gives rise to the cardiac crescent, which contributes to the development of left ventricle and atria. The secondary heart field is derived from the pharyngeal mesoderm, anterior to the cardiac crescent, and is marked by the expression of Islet1 (Isl1), a transcription factor of the LIM-homeo-domain family. During ESC differentiation, Isl1 protein is first detected around D6 and reaches its maximum at D8, and its expression can be used to isolate multipotent cardiovascular progenitors of the secondary heart field (Moretti et al., 2006). To determine whether Mesp1 promotes the specification of MCPs from the primary and/ or the secondary heart field, we monitored the expression of Isl1 following Mesp1 induction. In Mesp1 stimulated cells, Is/1 mRNA expression was increased 4 days after Dox addition (D6). This effect was only transient, and Isl1 expression returned to the control level at D8 (Figure 2F). Immunostaining of EBs revealed the precocious expression of IsI1 in Mesp1-stimulated cells in which EBs at D6 contained as many Isl1-positive cells as control cells 2 days later (Figure 2G). Immunostaining revealed the presence of cells that coexpressed IsI1, Flk1, and Nkx2-5 following Mesp1 induction, consistent with the specification of MCPs from the secondary heart field by Mesp1 (Figure S2B) (Moretti et al., 2006).

To determine whether Mesp1 promotes the fate of other cell types during ESC differentiation, we analyzed the expression of a panel of markers specific for a wide range of differentiated cells that are produced after 10 days of ESC differentiation (Keller, 2005). Besides promoting an increase in the expression of cardiovascular markers, Mesp1 also increased the expression of hepatic markers such as albumin, alpha-feto protein, and TCF1 (Figure 2H), consistent with known cell nonautonomouspromoting effect of cardiac cells during liver development (Zaret, 2000). Mesp1 also promoted the expression of striated muscle cell markers such as MyoD or Myogenin (Figure 2H), but this effect only appeared around day 10 (Figure 2H and data not shown) during the late stage of ESC differentiation potentially related to the later expression and function of Mesp1 in the presomitic and somitic mesoderm (Saga, 1998; Takahashi et al., 2005). Expression of markers for other mesodermal derivatives, such as hematopoieitic tissue (Gata1, Tie2), bone (runx2, Col1a1), endodermal, or ectodermal derivatives were unchanged or relatively decreased in Mesp1 stimulated cells (Figure 2H).

## Mesp1 Specifies Multipotent Cardiovascular Progenitor Cell Fate by an Intrinsic and Cell Autonomous Mechanism

To identify the cellular mechanisms by which Mesp1 promotes MCP specification, we first determined whether the addition of conditioned media (CM) from Mesp1-stimulated cells to control cells could recapitulate the cardiac-promoting effect of Mesp1 expression (Figure S3A). The daily addition of CM from Mesp1stimulated cells did not promote or accelerate cardiac differentiation in control cells (Figure S3B), indicating that Mesp1 does not promote cardiovascular specification by the secretion of soluble proteins. To validate these observations, we cocultured Mesp1-IRES-GFP EBs with EBs that expressed the red fluorescent protein DsRed (Figure S3C). While 80% EBs that expressed Mesp1 (GFP positive) showed beating zones at day 7, only 20% of neighboring EBs that expressed DsRed presented signs of cardiac contraction at this stage (Figure S3D). We quantified this effect by determining the expression of troponinT in cells expressing Mesp1 (GFP positive) and in control cells (DsRed) by FACS analysis (Figure S3E). At day 8, 18% of Mesp1 stimulated cells expressed troponinT, whereas only 4% of the DsRed cells of the neighboring coculture EBs expressed this marker, similar to control cells.

As a more rigorous test of an autonomous or nonautonomous mechanism, we generated chimeric EBs in which ESCs conditionally expressing Mesp1 and DsRed are mixed together to form EBs containing both cell types (Figure 3A). These chimeric EBs were stimulated with Dox, and cardiac differentiation (troponinT +) was measured in the Mesp1 (GFP+) or control (DsRed+)

Figure 2. Mesp1 Specifically Promotes Multipotent Cardiovascular Progenitor Cell Fate

<sup>(</sup>A) Expression of cardiovascular markers analyzed by RT-PCR after 10 days of differentiation in Mesp1 stimulated cells (black bars) versus unstimulated cells at the same time (white bars). These data show an enhancement cardiovascular markers following Mesp1 induction. Data represent mean ± SEM of four biologically independent experiments.

<sup>(</sup>B–E) (B) Immunostainings of EBs for myosin light chain 2v (mlc2v), (C) myosin light chain 2a (mlc2a), (D) VE-cadherin, and (E) smooth muscle actin (SMA). These immunostainings demonstrate acceleration and enhancement in atrial, ventricular, and vascular markers expression in Mesp1-stimulated EBs. Squares indicate areas of magnification for each condition represented on the right of the panel.

<sup>(</sup>F) Expression of *Islet1* as measured by RT-PCR following Mesp1 induction. Each result is normalized for the expression of unstimulated cells at the same time of differentiation. Data represent mean ± SEM of three biologically independent experiments.

<sup>(</sup>G) Immunostaining of Islet1 in EBs demonstrates a precocious expression of islet1 at day 6 in Mesp1-induced EBs.

<sup>(</sup>H) Expression of other mesodermal, endodermal, and neurectodermal markers analyzed by RT-PCR after 10 days of differentiation in Mesp1-stimulated cells (black bars) versus unstimulated cells at the same time (white bars). Data represent mean ± SEM of four biologically independent experiments.



## Figure 3. Mesp1 Promotes Cardiac Progenitor Cell Fate by an Intrinsic and Cell Autonomous Mechanism

(A) Schematic representation of chimeric EB experiment. Equivalent cell number of Mesp1-IRES-GFP cells or control GFP ES cells were mixed with DsRed expressing cells.

(B) TroponinT expression in Mesp1-expressing cells (GFP positive) or in control cells (DsRed positive) was measured by FACS analysis after 8 days of differentiation. Percentage of troponinT-positive cells is much higher in Mesp1stimulated cells than in DsRed stimulated cells or in DsRed cells that have been in contact with GFP cells, demonstrating that Mesp1 promotes cardiac specification mainly through a cell autonomous mechanism. Data represent mean ± SEM of three biologically independent experiments.

cells by FACS. At D8, the percentage of troponinT-positive cells was much higher in Mesp1-stimulated cells (15%) than in DsRed cells that had been in direct contact with Mesp1 stimulated cells (6%) or in DsRed cells than have been in direct contact with GFP cells (4%) (Figure 3B). These results showed that Mesp1 pro-

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motes cardiac specification through an intrinsic and cell autonomous mechanism.

#### Effect of Mesp1 on Cardiac Progenitor Cell Expansion

After 48 hr of Mesp1 induction, EBs were bigger and presented two times more cells than control EBs (Figures S4A and S4B). However, the growth advantage of Mesp1-stimulated cells was only transient as 3 days after Mesp1 induction, both Mesp1stimulated and control cells grew at the same rate (Figure S4B). FACS analysis 48 hr after Mesp1 induction revealed that Mesp1 induction did not significantly modify the cell-cycle profile of these cells (Figure S4C), but apoptosis was significantly reduced in Mesp1-stimulated cells (Figure S4D), suggesting that the transient growth advantage observed following Mesp1 induction is related to a transient inhibition of apoptosis. The small and transient cell growth advantage observed in Mesp1-stimulated cells contrasted with the major increase in cardiovascular differentiation induced by Mesp1, suggesting that Mesp1 promotes cardiovascular cell-fate specification through an instructive rather than selective mechanism.

## Mesp1 Directly Regulates the Expression of the Core Cardiac Transcriptional Machinery

To uncover the molecular mechanisms by which Mesp1 induced cardiovascular specification, we performed a genome-wide analysis of Mesp1-regulated genes. We determined which genes were regulated upon Mesp1 induction by at least 1.5-fold in two separate experiments. Mesp1 regulated a discrete set of genes (586 of 45101 probes), corresponding to 1.3% of the murine genome. Among the 423 unique annotated genes that were rapidly modulated upon Mesp1 induction, 276 were upregulated, whereas 148 were downregulated, suggesting that Mesp1 exerts its function by both positive and negative regulation of gene expression.

Cardiac morphogenesis and differentiation are governed by an evolutionarily conserved set of transcriptional factors that regulate the expression of genes implicated in morphogenesis and patterning during heart development as well as the genes required for cardiac terminal differentiation (Olson, 2006). Our microarray analysis revealed that Mesp1 induced the rapid upregulation of many, if not all, genes belonging to the core cardiac transcriptional machinery: *Hand2*, *Gata4*, *Gata6*, *Tbx 20*, or *Myocardin* (Table 1). In addition to these genes, we found that *Nkx2-5* and *Mef2c*, although not listed in our microarray analysis due to their low levels of expression, were also upregulated by RT-PCR (Figure 4A). Other genes playing important role during cardiovascular development, such as *Hey2* or *Foxc1* (Fischer et al., 2004; Kume et al., 2001), were also rapidly upregulated following Mesp1 induction (Table 1).

To study in more detail how Mesp1 regulates the expression of these key cardiac transcriptions factors, we investigated, by RT-PCR, the kinetics of their upregulation following Mesp1 expression. As early as 18 hr following Dox addition, only 6 hr after the appearance of Mesp1 in the nucleus of ESCs, expression of *Hand2*, *Myocardin*, *Gata4*, *FoxH1*, and *FoxC1* were upregulated by 2- to 15-fold (Figure 4A). For most of these genes, the maximum increase in gene expression occurred 24 hr following Mesp1 induction, although a sustained increase in the expression of *Hand2*, *Myocardin*, *Nkx2-5*, *Mef2c*, or *FoxC1* was still

	Upregulated Genes	Downregulated Genes
Transcription factors	Ripply2 (43.7), Cited 1 (36.8),* Trim9 (22.7), Foxl2os (17.9), Hey2 (13.8),* Otx1 (13.6), dHand (6.7),* Ebf2 (6.3), Lhfp (5.5), Snai1 (4.9),* Lef1 (4.3),* Nfatc1 (4.0),* Pdlim4 (4.0), Myocd (3.7),* Pdlim2 (3.5), Asxl3 (3.3), Foxc1 (3.2),* sVax1 (3.1), Twist1 (3.1),* Fli1 (2.9), Fosl2 (2.8), Klhl6 (2.8), Zeb1 (2.7), Ankrd6 (2.6),* Insm1 (2.6), Gata4 (2.4),* Hes6 (2.6), Spic (2.5), Hmga2 (2.5),* Gbx2 (2.3), Pdlim5 (2.3), Dmrta1 (2.3), Ankrd1 (2.3),* sFOG (2.3),* Hmgn3 (2.2), Dact1 (2.1), Zfp711 (2.1), Pbx1 (2.1), Zfp238 (2.1), Specc1 (2.1), Hdgfrp3 (2.1), Dachshund1 (2.1), Etv2 (2.0),* Tshz1 (2.0), Hoxd13 (2.0), Tox (1.9), Lmo1 (1.9), Tbx20 (1.9),* Creb3l2 (1.9), Tbx3 (1.9),* Lbx1 (1.9), Gata6 (1.8),* Phc2 (1.8), L3mbtl3 (1.8), Neurod1 (1.7), Foxh1 (1.7),* Dicer (1.7),* Tceal1 (1.7), Fhl1 (1.7),* Sap30l (1.6), Ppfibp1 (1.6), Zeb2 (1.6),* Cbx2 (1.6), Sox4 (1.6),* Smad1 (1.6),* and Pitx2 (1.6)*	T (8.5), Foxa2 (6.3), Sox17 (6.2), Ldb2 (4.6), Klhl4 (4.3), Gsc (3.3), Sp8 (3.1), Id2 (2.7), Eras (2.7), Mixl1 (2.4), Zic5 (2.4), Irf6 (1.9), Foxd3 (2.1), Bhlhb2 (2.1), Nr5a2 (1,9), Hopx (1.9), Tox3 (1.8), Nkx6.3 (1.8), Prdm1 (1.8), Tcfcp2l1 (1.8), Dmrt1 (1.8), Esrrb (1.7), Mycl1 (1.7), Mcf2l (1.7), Nr0b1 (1.7), Pycard (1.7), Ltbp4 (1.6), Mybl2 (1.6), Klf2 (1.6), and Pcaf (1.5)
Signaling	Notch: ligands: <i>Dll3</i> (12.3), <i>Dll1</i> (3.2), <i>Dlk1</i> (3.0); receptors: <i>Notch1</i> (2.5), <i>Notch4</i> (2.0), <i>Notch3</i> (1.6); downstream transcription factors: <i>Hey2</i> (13.8), <i>Hes6</i> (2.6)	
	Wnt: Wnt5a (6.5), Lef1 (4.3)	Frzb (3.1)
	FGF: <i>Fgf</i> 3 (3.5)	Fgf8 (5.5), Fgf5 (2.6), Fgf17 (1.9)
	TGF-b: Tgfb1i1 (3.4), Fstl1 (2.9)	Fst (5.1), Cer1 (3.9), Nodal (1.5)
	Ras: Rhob (2.6), Rasl11b (2.3), Rasgrp3 (21.3), Rragd (1.7)	Rab25 (2.2), Shb (2.8)

observed after 48 hr or 72 hr of Mesp1 stimulation (Figure 4A). Addition of low dose of Dox, which induced Mesp1 at a lower level than the peak of its endogenous expression (Figure S1E), also stimulates the expression of these transcription factors (Figure S5A), demonstrating that promotion of these genes was not due to supraphysiological level of Mesp1. The very rapid upregulation of these genes by Mesp1 strongly suggested that they represent direct Mesp1 target genes. Other genes, such as *Isl1* (Figure 2F), did not present their maximal expression until 96 hr following Mesp1 induction, suggesting an indirect mode of regulation of these genes by Mesp1.

To determine whether these cardiac transcription factors are direct Mesp1 target genes, we performed chromatin immunoprecipitation (ChIP) analysis following Mesp1 induction and determined, by PCR analysis, whether ChIP using anti-Mesp1 antibody (Ab) enriched for DNA fragments containing conserved putative Mesp1 binding sites (EBox). Quantitative PCR analyses were performed for DNA fragments showing different amounts of PCR products following ChiP using anti-Mesp1 and isotype control antibodies (Figures S5-S8). Hand2 gene contains many conserved EBox sites in several conserved regions of its promoter (Figure 4B). Our ChIP experiments demonstrated that multiple regions within the Hand2 promoter were enriched using Mesp1 Ab (Figure 4C and Figure S5B). The strongest enrichment (about 20-fold) was found within a genomic region located 2.8 kB upstream of the ATG, a genomic region corresponding to the previously identified cardiac enhancer of Hand2 (McFadden et al., 2000). We identified other DNA regions also enriched following Mesp1 ChIP (Figures 4B and 4C; Figure S5B), whereas several other regions containing a cluster of conserved EBox sites within Hand2 promoter were not significantly enriched by Mesp1 ChIP (Figure 4C and Figure S5B), suggesting that Mesp1 binds directly and specifically to different promoter regions of Hand2, encompassing the previously identified cardiac enhancer (McFadden et al., 2000). To validate these results using another assay, we tested the ability of different Hand2 enhancers to promote transactivation of a reporter construct by Mesp1. Mesp1 stimulated the expression of reporter constructs containing the two distal enhancers and the more proximal cardiac enhancer, while no stimulation was observed in reporters without enhancer or containing a Hand2 enhancer without conserved EBox (Figure S5F). We identified one DNA region within the proximal promoter of myocardin containing a conserved EBox site that was strongly enriched by ChIP using Mesp1 Ab (about 20-fold) (Figures 4D and 4E; Figure S5C). The Nkx2-5 promoter contained at least three regions enriched for Mesp1 binding (Figures 4F and 4G; Figure S5D). Two of them are located in genomic regions previously identified as enhancers that promote Nkx2-5 expression in the cardiac crescent and heart tube (Schwartz and Olson, 1999). Gata4 contained two genomic regions located in the proximal promoter strongly enriched following Mesp1 ChIP, whereas other regions containing conserved EBox sites and located more upstream were not enriched by Mesp1 Ab (Figures 4H and 4I; Figure S5E).

## Mesp-1 Represses the Expression of Genes Regulating Pluripotency, Early Mesoderm, and Endoderm Cell Fates

Our microarray analyses indicated that Mesp1 rapidly repressed the expression of several genes implicated in the maintenance of pluripotency such as *Id2* and *Eras* (Table 1) (Takahashi et al., 2003; Ying et al., 2003). We expanded our analysis by examining the expression of other key regulators of pluripotency by RT-PCR (Jaenisch and Young, 2008). Expression of *Nanog*, *Oct4*,



and *Sox2* were also downregulated upon Mesp1 induction, but less rapidly than *Eras* or *Id2*, suggesting they do not represent direct Mesp1 target genes (Figures 5A and 5B). Using immunos-taining, we found that Nanog disappeared more rapidly during ESC differentiation in Mesp1-stimulated cells compared to control cells (Figure 5C).

During early gastrulation, specification of the primitive streak to mesoderm and endoderm cell fate is tightly regulated temporally and spatially by specific transcription factors such as Brachyury, Sox17, Foxa2, and also by different extrinsic factors such as Wnts or Nodal (Tam et al., 2003; Tam and Loebel, 2007). The formation of cells of the primitive streak also occurs during ESC differentiation and gives rise to either mesoderm or endoderm cells (Murry and Keller, 2008). Our microarray analysis showed that Brachyury (T) and FGF8, which are expressed throughout the primitive streak, as well as Foxa2 and Sox17, which are expressed in the anterior primitive streak and control the specification of definitive endoderm lineages, were among the five most downregulated genes following Mesp1 induction (Table 1). Several other genes important for early mesoderm and endoderm specification, such as Nodal, Goosecoid, Cerberus, Follistatin, and FoxD3 (Tam and Loebel, 2007), were also downregulated following Mesp1 induction, suggesting that Mesp1 selectively represses genes implicated in the specification of other early mesoderm and endoderm cell fates (Table 1).

We used RT-PCR to investigate in more detail the kinetics of their transcriptional repression by Mesp1. *Brachyury, Sox17, FGF8, Foxa2, Gsc,* and *Cer1* were rapidly downregulated following Mesp1 expression as soon as 12 hr after Dox addition and reached their maximal downregulation 24 hr after Dox addition (Figure 5D). Among these genes, *Foxa2* was the most downregulated. Using immunostaining, we demonstrated that Foxa2 expression was indeed strongly downregulated in cells expressing Mesp1 (Figure 5E).

To determine whether Mesp1 directly controls the expression of these genes, we performed ChIP experiments following Mesp1 induction and determined, using PCR analysis, whether immunoprecipitated fragments contained conserved EBoxe sites. Our ChIP experiments revealed that Mesp1 IP enriched by about 20-fold DNA fragments containing one conserved EBox site located 5 kB upstream of the ATG of *Foxa2* (Figures 5F and 5G; Figure S6A). Our ChIP experiments showed that DNA fragments located 4.5 kB upstream of the ATG of *Gsc* and containing a cluster of 10 conserved EBox sites were enriched by 20-fold using anti-Mesp1 antibody (Figures 5H and 5I; Figure S6B). Our ChIP experiments revealed that Mesp1 IP enriched by about 10-fold DNA fragments located 4 kB upstream of the ATG of *Sox17* (Figures 5J and 5K; Figure S6C). Mesp1 ChiP enriched by 5-fold DNA fragments located 1.5 kB upstream of the ATG of *Brachyury* (Figures 5L and 5M; Figure S6D). The specific binding of Mesp1 to regions of genomic DNA located in *Sox17*, *Gsc*, *Foxa2*, and *Brachyury* revealed by our ChIP experiments, together with their very rapid downregulation following Mesp1 induction, strongly suggest that Mesp1 directly controls the repression of genes involved in the specification of the other cell types that arise during the early stages of gastrulation.

## Mesp1 Directly Regulates Multiple Components of the Canonical Notch and Wnt Signaling Pathways and Prime These Pathways toward Cardiac Commitment

Wnt and Notch signaling pathways are well known to regulate different aspects of cardiovascular differentiation from progenitor specification to cardiac and vascular cell terminal differentiation (Cohen et al., 2008; Gridley, 2007).

Our microarray analysis revealed that many components of the Wnt and Notch pathways were rapidly upregulated following Mesp1 expression (Table 1). All three delta ligands, three of the four Notch receptors, and two well-known downstream target genes of the Notch pathway (Hey2, Hes6) were upregulated following Mesp1 induction (Table 1). The promotion of Notch ligands and Notch target gene expression mediated by Mesp1 was relatively transient, peaked after 24 hr, and decreased to the basal level thereafter, whereas the increase in Notch1 expression was more sustained (Figure 6A). To investigate whether Notch signaling was necessary for the cardiovascular specification induced by Mesp1, we treated ESCs with N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a gamma-secretase inhibitor that prevents Notch activation (Geling et al., 2002) while at the same time inducing Mesp1 expression. DAPT did not profoundly reduce the number of beating EBs after 8 days of differentiation in control or Mesp1-stimulated cells (Figure 6B). However, immunostaining analysis showed that DAPT treatment from day 2 to day 4 caused a decrease in the total number of troponinT- and VE-Cadherin-positive cells in both control and Mesp1-stimulated cells (Figures 6C and 6D), suggesting that Notch influences MCPs specification and/ or early cardiovascular lineage commitment.

It has been recently suggested that Mesp1 promotes cardiac differentiation through the upregulation of Dkk1, a soluble Wnt inhibitor (David et al., 2008). Our microarray and RT-PCR analysis revealed that Mesp1 promoted the expression of *Lef1*, a transcription factor that relays canonical Wnt signaling, and *Wnt5a*, a ligand of the noncanonical Wnt pathway, and decreased the expression of *Wnt3a*, a ligand of the canonical Wnt pathway (Table 1 and Figure 6E). We did not detect any change in *Dkk1* expression following Mesp1 induction, neither by microarray nor by real time RT-PCR (Figure 6F). We could not detect any enrichment of Mesp1 bound to *Dkk1* promoter by ChIP in the DNA

#### Figure 4. Mesp1 Directly Promotes the Expression of the Core Cardiac Transcriptional Machinery

(A) Temporal expression of cardiovascular transcription factors following a transient Mesp1 induction. Results are normalized for the expression in Mesp1-unstimulated cells at the same time of differentiation and represent the mean ± SEM of three biologically independent experiments. These results demonstrate a rapid upregulation of these genes, already detectable as early as 12–18 hr post-Dox stimulation. (B, D, F, and H) Representation of genomic regions surrounding *Hand2* (B), *Myocardin* (D), *Nkx2-5* (F), and *Gata4* (H) genes. Untranslated regions are depicted in yellow. Exons are shown by wide blue lines and intron by thin blue lines. The previously described cardiac enhancers are highlighted in green (McFadden et al., 2000; Schwartz and Olson, 1999). Conserved EBox sites between human and mouse sequences and relative position of PCR fragments used to measure the enrichment following ChIP are represented by orange boxes. (C, E, G, and I) Quantification of DNA fragment enrichment by ChIP using anti-Mesp1 antibody relative to control isotype antibody as measured by RT-PCR for *Hand2* (C), *Myocardin* (E), *Nkx2-5* (G), and *Gata4* (I). Data represent mean ± SEM of four biologically independent experiments.



region recently identified (Figures S7A and S7B). We found, as previously reported (Kwon et al., 2007; Lindsley et al., 2006; Naito et al., 2006; Ueno et al., 2007), that Dkk1 addition during ESC differentiation profoundly inhibits cardiac differentiation as measured by the number of beating EBs (Figure 6G) and the number of troponinT-positive cells (Figures 6H and 6I). Addition of Dkk1 during Mesp1 induction also decreased significantly, but not completely, the cardiac promoting effect of Mesp1 (Figures 6G-6l). We confirmed the promoting effect of Wnt3a addition on Mesp1 expression (Figure S7C) (Ueno et al., 2007) and demonstrated that addition of Dkk1 from D2 to D4 profoundly inhibited Mesp1 expression (Figure 6J), suggesting that Wnt signaling can act upstream of Mesp1 expression and potentially explains why Wnt signaling is important during cardiovascular specification. Our results reinforce the prevailing notion that stimulation of canonical Wnt signaling is required for cardiac progenitor specification and/or expansion (Klaus et al., 2007; Kwon et al., 2007; Lindsley et al., 2006; Naito et al., 2006; Qyang et al., 2007; Ueno et al., 2007) but clearly demonstrated that Dkk1 is not a direct Mesp1 target gene and that Dkk1 is not responsible for the cardiac promotion mediated by Mesp1. Interestingly, while Dkk1 addition in the early stage of ESC differentiation inhibits cardiac specification, Dkk1 addition during the latter stage promotes cardiac differentiation, suggesting that Wnt signaling presents a biphasic effect during cardiac differentiation (Naito et al., 2006; Ueno et al., 2007; Yang et al., 2008).

## Mesp-1 Regulates Its Own Expression through a Complex Gene Regulatory Circuit

Mesp1 and Mesp2 are well known to repress each other's expression and possibly also their own expression (Kitajima et al., 2000). Positive and negative autoregulatory loops are common mechanisms during cell-fate specification, ensuring a sharp boundary of gene expression as well as transient gene expression (Alon, 2007). Our microarray analysis revealed that Ripply2, a well known direct negative regulator of Mesp2 expression (Kawamura et al., 2008; Morimoto et al., 2007), was the most upregulated gene (about 50-fold) following Mesp1 induction (Table 1). Ripply2 expression is barely detectable at day 3 of ESC differentiation, and its expression was very rapidly and strongly upregulated following Mesp1 induction (Figure 7A). We investigated, using ChIP experiments, whether Mesp1 directly binds to Ripply2 promoter. Our ChIP experiments revealed that Mesp1 Ab enriches, by 20-fold, DNA fragments located 6.5 kB upstream of the ATG of Ripply2 and containing a cluster of four conserved Ebox sites (Figures 7B and 7C; Figure S8A).

To determine whether Mesp1 regulates its own expression, we measured the endogenous level of *Mesp1* following Mesp1 induction by designing RT-PCR primers specific for the 3'UTR re-

gion of endogenous Mesp1 mRNA that do not amplify the Mesp1 mRNA of the inducible construct. Interestingly, Mesp1 expression initially stimulated its own expression, but this effect was only transient. After 24 hr following Dox addition, the endogenous expression of Mesp1 and Mesp2 were downregulated in Mesp1-stimulated cells (Figure 7D). The rapid and transient stimulation of Mesp1 expression following exogenous Mesp1 induction strongly suggested that Mesp1 first stimulated its own expression through a direct positive-feedback loop. We used ChIP experiments to determine whether Mesp1 binds directly to its own regulatory region. Our ChIP experiments revealed that Mesp1 Ab enriched for DNA fragments located 4.6 kB upstream of the transcription initiation site of Mesp1 (Figures 7E and 7F; Figure S8B), suggesting a direct autoregulation of Mesp1. The subsequent downregulation of Mesp1 expression suggested an indirect mechanism, possibly mediated by the increase of Ripply2 expression.

## DISCUSSION

## Mesp1 Acts Intrinsically to Promote Multipotent Cardiovascular Progenitor Specification from Undifferentiated Mesoderm

ESC differentiation constitutes both a powerful method to dissect the cellular and molecular mechanisms underlying cardiac-fate decision and a potential source of cells for future cardiac and vascular cellular therapy in humans (Keller, 2005; Martin-Puig et al., 2008). In this study, we demonstrated that Mesp1 acts in a cell-autonomous manner to promote the specification of MCPs from the primary and secondary heart fields. This was clearly demonstrated by the massive increase in cardiac, endothelial, and smooth muscle cells and the upregulation of markers common in both sources of cells (Nkx2-5, Gata4, Hand2, Tbx20, FoxH1) and specific for the primary (e.g., Tbx5) and the secondary (e.g., IsI1) heart field following Mesp1 induction.

Recently, it was proposed that Mesp1 induces cardiac specification through the secretion of Dkk1, a soluble Wnt inhibitor, suggesting that cardiac-fate specification induced by Mesp1 occurred through a cell-nonautonomous mechanism (David et al., 2008). However, the data presented here using conditioned medium from Mesp1 stimulated cells, coculture of Mesp1, and control EBs, as well as the codifferentiation of chimeric EBs, demonstrates unambiguously that cardiovascular specification induced by Mesp1 is mediated predominantly through an intrinsic and cell-autonomous mechanism. However, the small promotion of cardiac differentiation (about 15% of the total promotion of cardiac differentiation induced by Mesp1) observed in the non-Mesp1-expressing cells leaves open the possibility of a minor contribution of a non-cell-autonomous effect of Mesp1 during

#### Figure 5. Mesp1 Represses the Expression of Genes Regulating Pluripotency, Early Mesoderm, and Endoderm Cell Fates

(A and B). Expression of *Eras* and *Id2* (A), *Oct4*, *Nanog*, and *Sox2* (B) mRNAs following a transient Mesp1 expression determined by RT-PCR. Results are normalized for expression in unstimulated cells at the same time of differentiation. Note the more rapid downregulation of *Eras* and *Id2* expression (A) compared to *Oct4*, *Nanog*, and *Sox2* expression (B). Data represent mean ± SEM of three biologically independent experiments. (C) Immunostaining for Nanog on cytospins 48 hr after Dox stimulation (D4). (D) Temporal expression of early mesodermal and endodermal markers following Mesp1 induction using RT-PCR analysis. Results are normalized for the expression in Mesp1 unstimulated cells at the same time of differentiation. Data represent mean ± SEM of three biologically independent experiments. (E) Immunostaining for Foxa2 at D6 showed the downregulation in Foxa2 expression following Mesp1 induction. (F, H, J, and L) Representation of genomic regions surrounding *Foxa2* (F), *Gsc* (H), *Sox17* (J), and *Brachyury* (L) genes has been performed as described in Figure 4. (G, I, K, and M) Quantification of DNA fragments enrichment by ChIP using anti-Mesp antibody relative to control isotype antibody as measured by RT-PCR for *Foxa2* (G), *Gsc* (I), *Sox17* (K), and *Brachyury* (M). Data represent mean ± SEM of four biologically independent experiments.



Figure 6. Mesp1 Directly Regulates Multiple Components of the Canonical Notch and Wnt Signaling

(A) Temporal expression of *DII1*, *DII3*, *DII4*, *Notch1*, *Notch4*, *Hes6*, and *Hey2* following Mesp1 expression as determined by RT-PCR analysis. Results are normalized for the expression in Mesp1 unstimulated cells at the same time of differentiation. Data represent mean ± SEM of three biologically independent experiments.
(B) Percentage of beating EBs at day 8 (D8) following the administration of DAPT, an inhibitor of Notch activation, during day 2 (D2) and day 3 (D3) of ESC differentiation. Data represent mean ± SEM of three biologically independent experiments.

cardiac cell-fate specification. Our data are consistent with the in vivo chimeric studies showing that *Mesp1/2* null cells cannot generate cardiac cells despite the presence of wild-type cells, demonstrating the cell autonomous role of Mesp1/2 during mouse cardiac development (Kitajima et al., 2000).

## Mesp1 Directly Promotes the Expression of Key Components of the Core Cardiac Transcriptional Machinery

The core cardiac transcriptional machinery is composed of an evolutionarily conserved set of transcription factors that reinforce each other's expression and can stimulate alone or in combination the expression of genes required for proper cardiac development (Davidson and Erwin, 2006; Olson, 2006). Little is known about the upstream factors that initiate expression of these key cardiac transcription factors during development, resulting in their coregulated expression in the cardiac crescent of the primary and secondary heart fields. Our study demonstrates that Mesp1 rapidly and strongly stimulated the expression of Hand2, Myocardin, Nkx2-5, Gata4, Mef2c, Tbx20, or FoxH1. Our ChIP experiments showed that Mesp1 directly binds the previously described cardiac enhancers of Hand2 and Nkx2-5 (McFadden et al., 2000; Schwartz and Olson, 1999) and in various regulatory regions of the proximal promoter of Myocardin and Gata4. The strength of Mesp1 binding to the regulatory regions of these genes correlates well with the importance and the kinetics of their upregulation following Mesp1 expression, strongly suggesting that Mesp1 directly regulates their transcription. It has recently been shown that in ESCs in which Mesp1 has been knockdown, Nkx2-5 and Gata4 are no longer upregulated during differentiation, suggesting that Mesp1 is required for their upregulation (David et al., 2008). Our results place Mesp1 at the top of the transcriptional network that regulates cardiac differentiation by directly coordinating the expression of the majority of key cardiac transcription factors at the right place and at the right time (Figure 7G). A similar function of Mesp1, in coordinating cardiac specification by regulating cardiac gene expression, has also been suggested to occur during heart development in Ciona intestinalis, one of the most primitive chordates (Davidson et al., 2005). Clearly, further studies are needed to more precisely determine the Mesp1-binding sites to these regulatory regions and to determine the in vivo and the evolutionary significance of our findings during vertebrate cardiac specification.

## Mesp1 Directly Represses the Transcription of Genes Regulating Alternate Cell Fates

Developmental processes are dynamic in nature and always progress. Mesp1 downregulated the expression of about a hundred genes. The genes downregulated by Mesp1 included many genes involved the specification of early mesoderm and endoderm cell fates (Figures 5D and 7G). Mesp1 directly downregulated the expression of Brachyury and FGF8, which both act during early primitive streak specification, as well as Foxa2, Sox17, Gsc, Nodal, and Cer1, which all function during endoderm specification (Tam et al., 2003; Tam and Loebel, 2007). The temporal analysis of this gene expression following Mesp1 induction demonstrates that some genes (e.g., Brachyury, Foxa2, Gsc, and Sox17) were already strongly repressed only a few hours after the presence of Mesp1 in the nucleus (Figure 5D). These data, together with the direct demonstration that Mesp1 is bound to their regulatory region, strongly suggest that Mesp1 directly controls the transcription of these genes. The in vivo relevance of our findings is illustrated by the expanded and increased expression of Fgf8, Foxa2, Cereberus, Goosecoid, and Nodal in embryos deficient for Mesp1/2, demonstrating that Mesp1/2 negatively controlled expression of these key developmental genes in vivo as well (Kitajima et al., 2000; Saga et al., 1999). The repression of these early mesodermal and endodermal genes by Mesp1 may ensure that Mesp1 specifically, unidirectionally, and irreversibly induces the promotion of cardiovascular specification and inhibits the acquisition of other possible cell fates during this developmental stage.

# Mesp1 Negatively Regulates Its Expression through a Complex Gene Regulatory Network

We demonstrated that Mesp1 rapidly but transiently stimulated its own endogenous expression, probably through a direct mechanism as suggested by our ChIP experiments. This transient increase in Mesp1 expression is followed by a sustained and profound downregulation of its own endogenous expression. The most upregulated gene following Mesp1 stimulation is Ripply2, a transcriptional corepressor (Kawamura et al., 2008). Ripply2 was recently shown to be a direct target gene of Mesp2 as well as a negative regulator of Mesp2 expression in vivo (Morimoto et al., 2007). Our ChIP experiments demonstrated that Ripply2 is also a direct Mesp1 target gene, and our temporal expression analysis following Mesp1 stimulation showed that the profound and sustained upregulation of Ripply2 following Mesp1 stimulation is correlated with the secondary repression of Mesp1 expression, suggesting that Ripply2 may mediate the secondary negative autoregulatory loop of Mesp1 expression. Using Mesp1 lacZ knockin mice, Saga and colleagues demonstrated that Mesp1 negatively regulates its own expression in vivo (Saga et al., 1999). The positive and then negative autoregulation of Mesp1 presumably ensures that Mesp1 acts as a gene regulatory switch during cardiovascular specification during embryonic development and ESC differentiation.

<sup>(</sup>C and D) Immunostainings at day 8 for troponinT (C) and VE-cadherin (D) following DAPT administration during D2 and D3. DAPT treatment during D2-D3 decreases the number of troponinT- and VE-cadherin-positive cells.

<sup>(</sup>E and F) Temporal expression of *Lef1*, *Wnt3a* and *Wnt5a* (E), and *Dkk1* (F) following Mesp1 expression. Results are normalized for expression in unstimulated cells at the same time of differentiation. Data represent mean ± SEM of three biologically independent experiments.

<sup>(</sup>G) Percentage of beating EBs determined at D8 following addition of Dkk1 (D2-D3). Addition of Dkk1 resulted in an inhibition of cardiac differentiation in both control and Mesp1-stimulated cells. Data represent mean ± SEM of three biologically independent experiments.

<sup>(</sup>H and I) Inhibition of cardiac differentiation (troponinT) following Dkk1 administration, determined by FACS analysis (H) and immunostaining for troponinT (I) at day 10.

<sup>(</sup>J) Repression of *Mesp1* expression following Dkk1 administration from D2 to D3 in unstimulated cells, as measured by RT-PCR at D3. Results are normalized for *Mesp1* expression in untreated cells at the same day. Data represent mean ± SEM of three biologically independent experiments.

Cell Stem Cell Mesp1 Functions in Cardiovascular Specification





(A) Temporal expression of *Ripply2* following Mesp1 expression as determined by RT-PCR analysis. *Ripply2* expression is strongly and rapidly upregulated following Mesp1 induction. Results are normalized for *Ripply2* expression in unstimulated cells at the same time of differentiation. Data represent mean ± SEM of three biologically independent experiments.

## Conclusions

Our study provides important insights into the molecular mechanisms that promote the specification of MCPs from ESCs. We demonstrate that Mesp1 acts as a key molecular switch during this process, residing at the top of the hierarchy of the cardiovascular transcriptional network and stimulating the coordinated expression of the main transcription factors necessary for cardiovascular development (Figure 7G). Our genome-wide transcriptional analysis of Mesp1 target genes provides a comprehensive analysis of the earliest molecular mechanisms controlling cardiovascular commitment, which will constitute a framework for further exploration of the complex transcriptional network involved in cardiovascular progenitor specification. In addition, our study provides a robust method for generating cardiovascular cells during ESC differentiation and opens new avenues for cardiac cellular therapy in humans.

## **EXPERIMENTAL PROCEDURES**

#### Tetracycline-Inducible Mesp1 ESC Line

Murine Mesp1 ORF was cloned with a C terminus  $3 \times$  flag sequence and an IRES-GFP. This constructed was electroporated in A2Lox cells (M.I. and M.K., unpublished data). Further details are documented in the Supplemental Data.

#### **ESCs Culture and Differentiation**

A2Lox ESCs were maintained on irradiated MEFs. Medium composition and hanging drop differentiation protocol are described in the Supplemental Data. When indicated, RmDKK1 (R&D) was used at a final concentration of 600 ng/ml and DAPT at a final concentration of 2.5  $\mu$ M.

#### **RNA Isolation and Microarray Analysis**

Uninduced and induced Mesp1 cells were collected 24 hr postdoxycyclin stimulation. RNAs were purified using Absolutely RNA kit (Stratagene), labeled, and hybridized on a mouse genome 430 2.0 array. Raw microarray images were quantified using Gene ChIP Operating Software (GCOS, Affymetrix). Analysis parameters are described in the Supplemental Statistical Analysis.

#### **Reverse Transcription and Quantitative PCR**

Reverse transcription and quantitative PCR procedures are described in the Supplemental Experimental Procedures.

#### Immunofluorescence Analysis

Staining procedures and antibodies used are described in the Supplemental Experimental Procedures.

#### **Chromatin Immunoprecipitation Assay**

ChIP assays were performed in embryoid bodies at 36 hr after Dox induction. For all ChIP experiments, the anti-Flag M5 or the related isotype was used for the immunoprecipitation step as described in the Supplemental Data. Quantitative PCR analyses were performed for DNA fragments showing different amount of PCR products following ChiP using anti-Mesp1 and isotype control antibodies. Relative occupancy values were calculated by the ratios of amount of antibody-immunoprecipitated DNA over that of the isotype sample. Detailed procedures and a list of primers used for ChIP-PCR are documented in the Supplemental Data.

#### Statistical Analysis

Statistical analysis is described in detail in the Supplemental Data.

#### SUPPLEMENTAL DATA

The Supplemental Data include eight figures, Supplemental Statistical Analysis, Supplemental Experimental Procedures, two tables, and two movies and can be found with this article online at http://www.cellstemcell.com/cgi/ content/full/3/1/69/DC1/.

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(B) Representation of the genomic region surrounding the Ripply2 gene has been performed as described in Figure 4.

(C) Quantification of DNA fragments in the *Ripply2* gene enriched by ChIP using anti-Mesp antibody relative to control isotype as measured by RT-PCR. Data represent mean ± SEM of four biologically independent experiments.

(D) Temporal expression of endogenous *Mesp1* and *Mesp2* following Mesp1 expression by RT-PCR analysis. Results are normalized for expression in unstimulated cells at the same time of differentiation. Endogenous *Mesp1* transcript is specifically detected by PCR of the 3' UTR region of *Mesp1*, which is not presented in the inducible construct. Note the biphasic effect of Mesp1 on its endogenous expression. Data represent mean ± SEM of three biologically independent experiments.

(E) Representation of the genomic region surrounding the Mesp1 gene.

(F) Quantification of DNA fragments enrichment by ChIP using anti-Mesp antibody relative to control isotype measured by RT-PCR for *Mesp1*. Data represent mean ± SEM of four biologically independent experiments.

(G) Model of Mesp1 functions during multipotent cardiovascular progenitor specification.

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