

## Development of Bipotent Cardiac/Skeletal Myogenic Progenitors from MESPI+ Mesoderm

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### SUMMARY

The branchiomic skeletal muscles co-evolved with new chambers of the heart to enable predatory feeding in chordates. These co-evolved tissues develop from a common population in anterior splanchnic mesoderm, referred to as cardiopharyngeal mesoderm (CPM). The regulation and development of CPM are poorly understood. We describe an embryonic stem cell-based system in which MESPI drives a PDGFRA+ population with dual cardiac and skeletal muscle differentiation potential, and gene expression resembling CPM. Using this system, we investigate the regulation of these bipotent progenitors, and find that cardiac specification is governed by an antagonistic TGFβ-BMP axis, while skeletal muscle specification is enhanced by Rho kinase inhibition. We define transcriptional signatures of the first committed CPM-derived cardiac and skeletal myogenic progenitors, and discover surface markers to distinguish cardiac (PODXL+) from the skeletal muscle (CDH4+) CPM derivatives. These tools open an accessible window on this developmentally and evolutionarily important population.

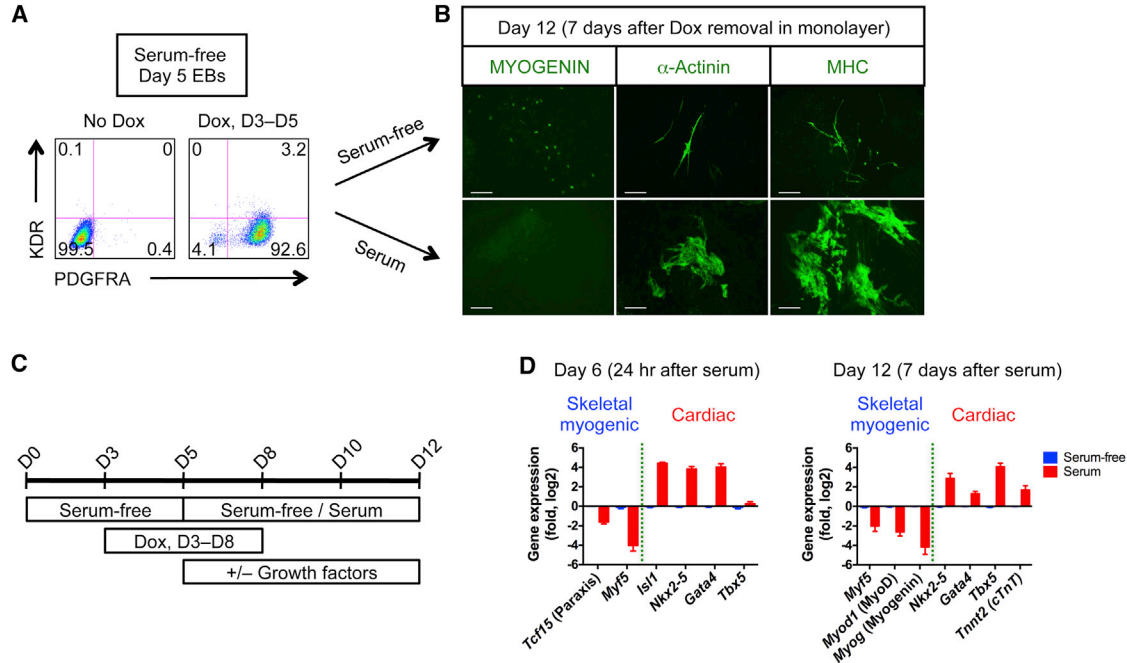
### INTRODUCTION

Cardiac and skeletal myogenesis both generate striated muscle, but are traditionally thought of as two separate processes with distinct developmental anlagen. However, recent work suggests the existence of at least one bipotent anlage for cardiac and skeletal muscle: the common progenitor population that leads to the formation of both the head muscles and the second heart field (Lescroart et al., 2010; Nathan et al., 2008; Tirosh-Finkel et al., 2006), and which is unique to chordates (Diogo et al., 2015). Somite-derived myogenesis depends on PAX3 to initiate the core myogenic network (Tajbakhsh et al., 1997), but the head muscles do not derive from a PAX3+ progenitor, instead requiring TBX1 and PITX2 (Sambasivan et al., 2009). These genetic differences between head and trunk muscles may have functional consequences, as several muscular dystrophies have differential severity in craniofacial skeletal muscles compared with trunk muscles (e.g., facioscapulohumeral muscular dystrophy shows greater involvement of facial muscles, while Duchenne muscular dystrophy shows less). Cranial myogenesis has been much less investigated than somite-derived myogenesis.

Cranial mesoderm can be divided into three regions: pre-chordal mesoderm, cranial paraxial mesoderm, and cardio-

pharyngeal mesoderm (CPM). CPM, also known as lateral splanchnic mesoderm, is the only embryonic population with both cardiac and skeletal myogenic potential, giving rise to second heart field and distal facial skeletal muscles (e.g., digastric, mylohyoid, and stylohyoid) (Lescroart et al., 2010; Tirosh-Finkel et al., 2006). This developmental intimacy may explain why cardiac and craniofacial congenital defects are often linked, with DiGeorge syndrome being a prominent example (Hutson and Kirby, 2003).

The transcription factor MESPI is essential to mesoderm patterning (Saga et al., 1999). In the ascidian *Ciona*, cells expressing the homolog *Mesp* give rise to the trunk ventral cells, which subsequently develop into cardiac and skeletal progenitors (Satou et al., 2004). The *Ciona* trunk ventral cells thus act as the ascidian CPM (Razy-Krajka et al., 2014). In the mouse, MESPI is the earliest acting factor in heart development (Saga et al., 1999). We recently reported that MESPI also promotes other mesoderm lineages including hematopoietic and skeletal myogenic (Chan et al., 2013). Lineage-tracing studies further revealed that *Mesp1*+ cells contribute to the muscle stem cell pool of the head (Chan et al., 2013; Harel et al., 2009). While the cardiac effects of MESPI are well studied (Bondue et al., 2008, 2011; David et al., 2008; Lindsley et al., 2008), the mechanisms governing the differentiation of these *Mesp1*-marked bipotent CPM progenitors remain poorly understood.



**Figure 1. MESP1 Promotes Cardiac or Skeletal Myogenic Differentiation Depending on Serum-Factor Signaling**

(A) MESP1 induced a KDR<sup>−</sup> PDGFRA<sup>+</sup> population in day-5 EBs cultured in serum-free condition.

(B) These putative paraxial mesoderm cells gradually acquired a skeletal myogenic fate that was tubular, MYOGENIN<sup>+</sup>,  $\alpha$ -actinin<sup>+</sup>, and myosin heavy chain (MHC)<sup>+</sup> by day 12 (top row). Serum supplement from day 5 produced cardiac cells that were planar, MYOGENIN<sup>−</sup>,  $\alpha$ -actinin<sup>+</sup>, and MHC<sup>+</sup> (bottom row). Images are representative of five independent experiments. Scale bar represents 100  $\mu$ m.

(C) Scheme depicting the protocol used to evaluate the effects of various treatments on MESP1-induced cardiac versus skeletal myogenic differentiation.

(D) Quantitative RT-PCR analysis showing that the addition of serum downregulated skeletal myogenic genes (*Tcf15*, *Myf5*, *Myo1*, *Myog*) and upregulated cardiac genes (*Isl1*, *Nkx2-5*, *Gata4*, *Tbx5*, *Tnnt2*) as early as 24 hr (left), and also after 7 days (right) ( $n = 12$ , from four independent experiments). Mean  $\pm$  SEM is shown.

See also [Figure S1](#) and [Tables S1](#) and [S2](#).

Here we report that under serum-free conditions, MESP1 induction in embryonic stem (ES) cells produces PDGFRA<sup>+</sup> progenitors with dual cardiac/skeletal myogenic potential, functionally resembling CPM. We further demonstrate the robustness of this MESP1-induced system for identifying pathways that enhance the cardiac/skeletal myogenic bifurcation and discovering cell-surface proteins as markers to distinguish these two lineages.

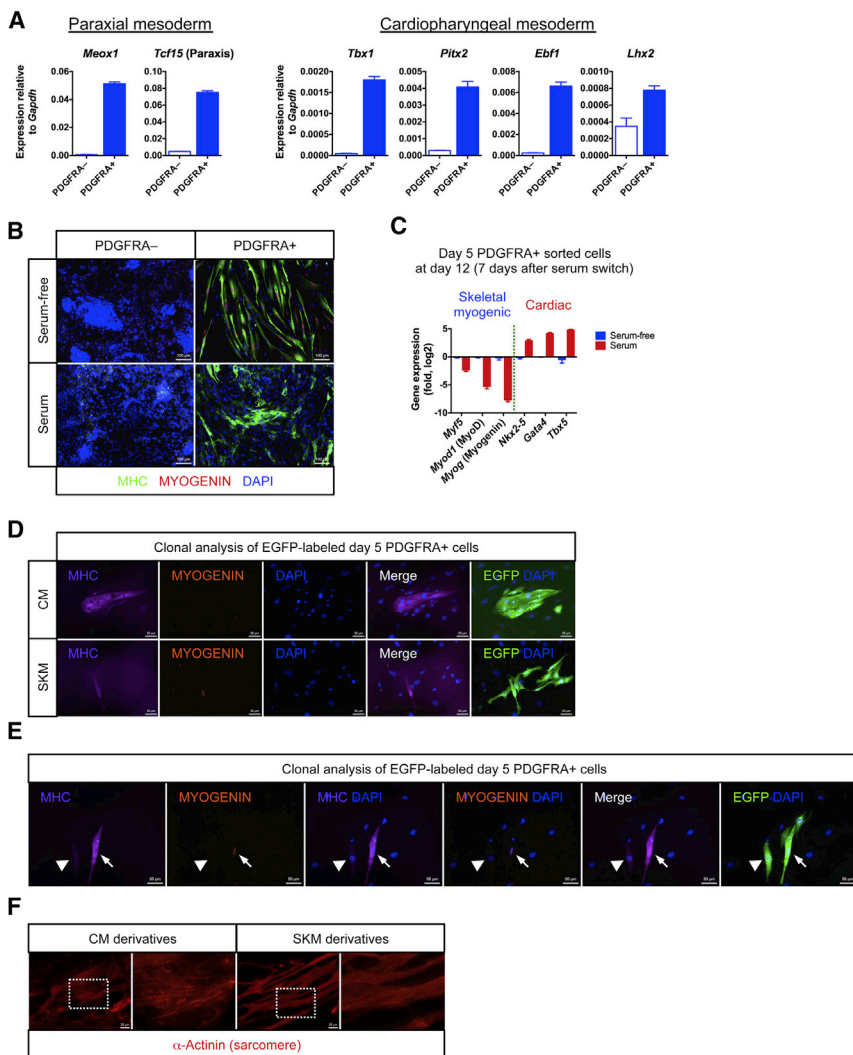
## RESULTS AND DISCUSSION

### MESP1 Induces Cardiac or Skeletal Myogenic Differentiation Depending on Serum-Factor Signaling

We have previously developed a mouse ES cell line in which MESP1 expression can be tightly regulated by the addition of doxycycline (Dox) (Chan et al., 2013). In embryoid bodies (EBs) cultured in serum-free medium, mesoderm does not form, as evidenced by the absence of

KDR (Flk-1) and PDGFRA (PDGFR $\alpha$ ) (Figure 1A, No Dox). In contrast, MESP1 induction from days 3–5 of serum-free culture generated a KDR<sup>−</sup> PDGFRA<sup>+</sup> population (Figure 1A, Dox). When attached and continued in serum-free culture, skeletal myocytes emerged by day 12 (Figure 1B, top row). If serum was included from the start, MESP1 induction led to cardiac differentiation instead (Chan et al., 2013). We next performed differentiation in serum-free conditions, but added back serum at day 5. Surprisingly, this converted differentiation toward cardiac instead of skeletal myogenic (Figure 1B, bottom row). We tested different windows of MESP1 induction to map the window of maximal skeletal myogenic activity and found that the day 3–8 window was optimal (Figure S1A); thus this window was used for subsequent studies (Figure 1C).

Gene expression analysis revealed that within 24 hr of serum addition, paraxial mesoderm (*Tcf15*, paraxis) and skeletal myogenic genes (*Myf5*) were downregulated while cardiac genes (*Isl1*, *Nkx2-5*, and *Gata4*) were upregulated



(F) Sarcomeric structures were observed in cardiac (left) and skeletal myogenic derivatives (right). Areas depicted by the white dotted rectangle are magnified for clarity. Scale bar represents 20  $\mu\text{m}$ . See also [Figure S2](#) and [Tables S1](#) and [S2](#).

([Figure 1D](#), left panel). Further culture to day 12 potentiated these effects, with enhanced *Tnnt2* (cardiac troponin T) and reduced *Myod1* (MyoD) and *Myog* (myogenin) expression ([Figure 1D](#), right panel). We also tested serum add-back over different windows and found add-back from day 5 to be the most potent ([Figure S1B](#)). Therefore, both cardiac and skeletal myogenic progenitors derive from a common MESP1+ population, and the cardiac lineage choice can be driven by factors present in serum.

### MESP1-Induced PDGFRA+ Cells Functionally Resemble CPM at the Single-Cell Level

We next addressed whether specification of these two lineages is pre-determined prior to, or occurs after, MESP1

### Figure 2. MESP1-Induced PDGFRA+ Progenitors Give Rise to Cardiac or Skeletal Myogenic Derivatives at a Single-Cell Level

(A) Paraxial mesoderm genes (*Meox1*, *Tcf15*) and CPM genes (*Tbx1*, *Pitx2*, *Ebf1*, *Lhx2*) were enriched in MESP1-induced day-5 PDGFRA+ cells cultured in serum-free condition ( $n = 6$ , from two independent experiments). Mean  $\pm$  SEM is shown.

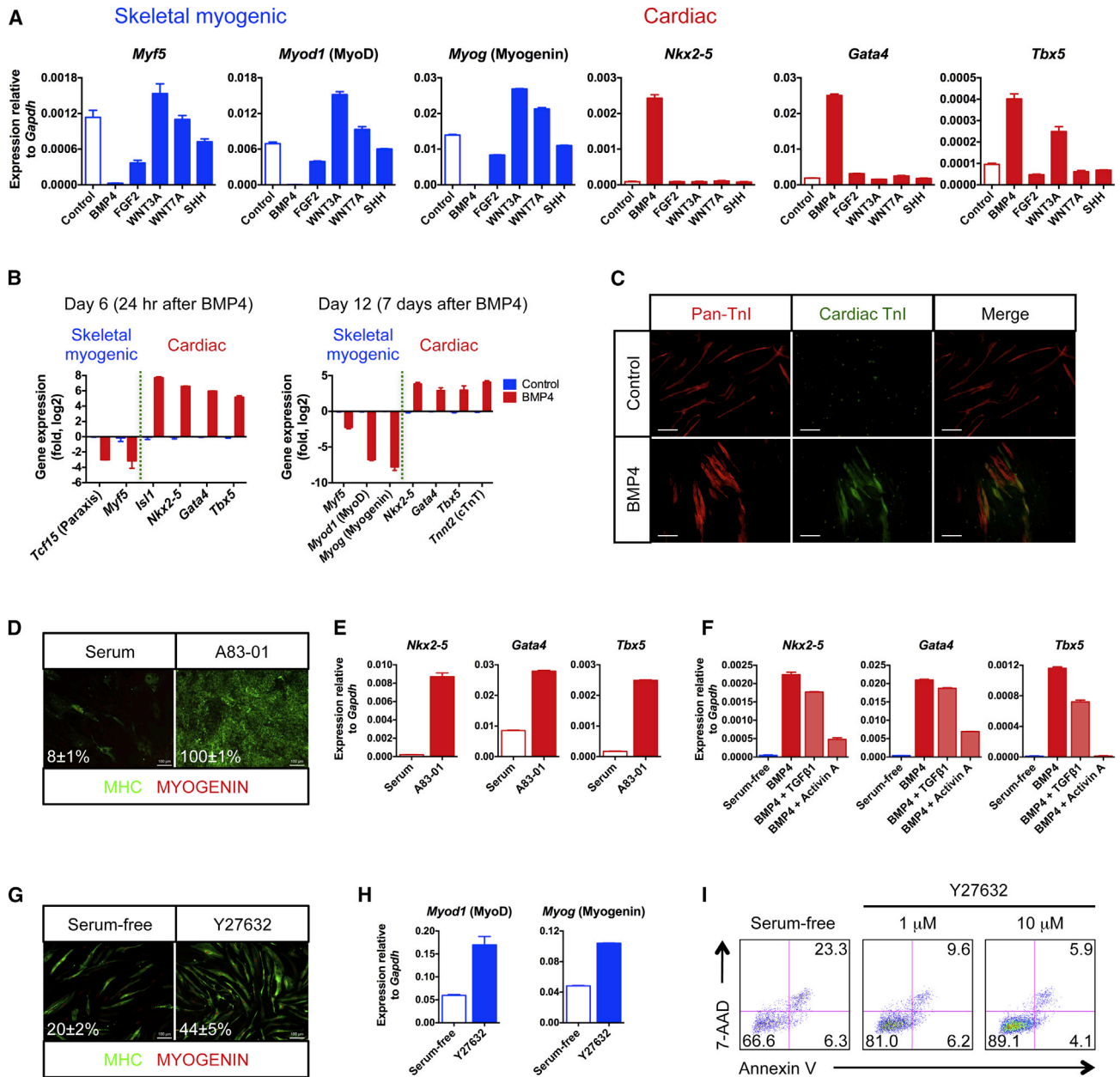
(B) Immunostaining shows that day-5 PDGFRA+ cells predominantly acquire a skeletal myogenic fate (MHC+ MYOGENIN+) in serum-free conditions or a cardiac fate (MHC+ MYOGENIN-) in serum-containing condition by day 12, but PDGFRA- cells do not acquire either fate. Images are representative of three independent experiments. Scale bar represents 100  $\mu\text{m}$ .

(C) Quantitative RT-PCR shows that the default skeletal myogenic differentiation of day-5 PDGFRA+ cells under serum-free conditions switches to cardiac in the presence of serum ( $n = 6$ , from two independent experiments). Mean  $\pm$  SEM is shown.

(D and E) Clonal analysis of MESP1-induced PDGFRA+ cells. (D) Single EGFP-labeled MESP1-induced PDGFRA+ cells gave rise to cardiac (CM) or skeletal myogenic (SKM) derivatives. Images are representative of four independent experiments. (E) A single MESP1-induced PDGFRA+ cell differentiated into both cardiac (arrowhead) and skeletal myogenic (arrow) lineages. Cells were grown in serum-free conditions supplemented with Y27632, conditions under which skeletal myogenic differentiation predominates. Scale bar represents 50  $\mu\text{m}$ .

induction. A clear positive answer could be obtained if a single cell were found to give rise to progeny of both cardiac and skeletal myogenic cells. We first confirmed that the PDGFRA+ fraction was enriched for paraxial mesoderm genes such as *Meox1* and *Tcf15* ([Figure 2A](#)). Importantly, transcription factors pertaining to CPM, including *Tbx1*, *Pitx2*, *Ebf1*, and *Lhx2*, were indeed enriched in PDGFRA+ cells ([Figure 2A](#)). Upon further culture in serum-containing or serum-free conditions, PDGFRA+ cells differentiated into cardiac or skeletal myogenic derivatives, respectively, but PDGFRA- cells did not ([Figures 2B](#) and [2C](#)).

Single PDGFRA+ cells had poor survival upon fluorescence-activated cell sorting (FACS) and rarely produced colonies, but cloning efficiency was increased by culture on a



**Figure 3. Signaling Environment Governs Cardiac versus Skeletal Myogenic Differentiation of MESP1+ Mesoderm**

(A) Screening of modulators of signaling pathways involved in myogenesis revealed that BMP4 decreased skeletal myogenic gene expression and increased cardiac gene expression by day 12 ( $n = 3$ , technical replicates).

(B) BMP4 promoted cardiac at the expense of skeletal myogenic differentiation as early as 24 hr (left) and after 7 days (right) ( $n = 3$ , technical replicates).

(C) Immunostaining using pan-troponin I (for both skeletal and cardiac) and cardiac-specific troponin I verified that MESP1 induced pan-TnI+ cTnI- skeletal myogenic cells in serum-free conditions (control), whereas BMP4 produced pan-TnI+ cTnI+ cardiac cells instead.

(D and E) ALK4/5/7 inhibition by A83-01 promoted cardiac differentiation, as shown by immunostaining (D) and quantitative RT-PCR (E) ( $n = 3$ , technical replicates). Efficiency of differentiation was determined by staining for MHC and DAPI, and analyzing images with G-Tool (Ippolito et al., 2012) ( $n = 3$ ). Images are representative of three independent experiments.

(F) TGFβ/activin-BMP antagonism, as shown by TGFβ1 and activin A diminishing the pro-cardiac effect of BMP4 ( $n = 3$ , technical replicates).

(legend continued on next page)





mouse embryonic fibroblast (MEF) feeder layer. To distinguish MESP1-induced PDGFRA<sup>+</sup> cells from MEFs, we engineered an EGFP-labeled MESP1-inducible ES cell line (Figures S2A–S2C). Although most cells committed to one or the other fate (Figure 2D), we observed that cardiac and skeletal myogenic progenitors were occasionally found within the same colony (Figure 2E, arrowhead and arrow, respectively), demonstrating that a single PDGFRA<sup>+</sup> cell had the potential to give rise to both lineages.

### MESP1-Induced CPM Cardiac Derivatives Have Second Heart Field Characteristics

We further evaluated the maturity of MESP1-induced CPM derivatives. Using optimal pro-cardiac or pro-skeletal myogenic culture conditions, CPM derivatives with clear sarcomeric structures were readily observed (Figure 2F). The maturity of CPM cardiac derivatives was also confirmed by observation of spontaneous rhythmic contractions (Movie S1).

We next compared the cardiac differentiation efficiency of the current CPM protocol with the previously reported MESP1-induced serum-throughout protocol (Chan et al., 2013). Whereas a simple serum add-back regimen was suboptimal in promoting cardiac differentiation, supplemental ALK4/5/7 inhibition (by A83-01, see below) in PDGFRA<sup>+</sup>-sorted cells greatly induced the expression of cardiac genes comparable with the serum-throughout protocol (Figure S2D). The CPM protocol thus efficiently generates cardiac progenitors.

During development, cardiac progenitors derived from CPM constitute the second heart field which populates both atria. Indeed, MESP1-induced CPM cardiac derivatives predominantly expressed atrial specific genes, including transcription factor (*Nr2f2*, COUP-TFII), myosin (*Myl2*, *Mlc-2a*), ion channel (*Kcnj3*, *K<sub>ir</sub>3.1*) and secretory peptide (*Nppa*, ANP) (Figure S2E). Conversely, ventricular specific genes such as *Hand1* and *Myl2* (*Mlc-2v*) were enriched in cardiac progenitors produced using the conventional protocol (Chan et al., 2013) (Figure S2F).

### A Subset of BMPs Promotes Cardiac at the Expense of Skeletal Myogenic Differentiation of MESP1+ Mesoderm

CPM is marked by *Mesp1* lineage tracing (Saga et al., 1999) and is known to be regulated by bone morphogenetic protein (BMP) signaling (Tzahor et al., 2003). To gain insight into signaling pathways that may regulate the cardiac

versus skeletal myogenic switch in this in vitro system, and to determine its relevance to CPM, we screened a panel of growth factors with known contributions to cardiac or skeletal myogenesis during embryo development. Among them, BMP4 downregulated skeletal myogenic genes and upregulated cardiac genes by day 12 (Figure 3A). This bias was apparent as early as 24 hr and was sustained for 7 days (Figures 3B and 3C). Thus, like CPM, which lineage-traces to *Mesp1*+ founders, the ES cell-derived MESP1-induced PDGFRA<sup>+</sup> population responds to BMP4 signaling by selecting the cardiac fate over the skeletal myogenic fate.

As BMP4 belongs to the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily, we next investigated the activities of the entire family (Figure S3A). BMP2, which is highly related to BMP4, behaved similarly, and BMP9 and 10 were somewhat effective. Interestingly, several other BMP members inhibited skeletal myogenesis but had minimal pro-cardiac response. Factors from branches outside of the BMP subfamily, including TGF $\beta$ 1/2/3, activin A, and nodal, did not promote cardiac specification.

### Inhibition of ALK4/5/7 and ROCK Improves Generation of MESP1+ Mesoderm Derivatives

To gain further insight into the cardiac-skeletal muscle lineage decision, we screened 80 inhibitors of defined stem cell-related signaling pathways for the ability to modulate differentiation of MESP1-induced day-5 PDGFRA<sup>+</sup> cells (Table S3). Under serum-replete conditions, we found that SB431542, A83-01, GW788388, and RepSox further enhanced cardiac differentiation (Figures 3D and S3B). Notably, these all suppress the TGF $\beta$ /activin/nodal signaling pathway by inhibiting ALK4/5/7 (TGF $\beta$  type I receptors), and, as exemplified by A83-01, expression of cardiogenic genes was upregulated (Figure 3E). As TGF $\beta$ 1/2/3, activin A, and nodal by themselves provide no bias toward cardiac or skeletal myogenesis (Figure S3A), we speculate a probable TGF $\beta$ 1/activin A-BMP4 pathway antagonism (Candia et al., 1997) in which the pro-cardiac effect of ALK4/5/7 inhibition might be due to a potentiation of BMP4 signaling. Indeed, BMP4-mediated cardiac gene up-regulation was reduced by TGF $\beta$ 1 and more profoundly so by activin A (Figure 3F).

We also found five compounds that further enhanced skeletal myogenic differentiation in the serum-free condition: Y27632, SR3677, GSK429286, thiazovivin (all Rho-associated kinase [ROCK] inhibitors), and blebbistatin (inhibitor of myosin II ATPase, a downstream target of

(G and H) ROCK inhibition by Y27632 promoted skeletal myogenic differentiation, as shown by immunostaining (G) and quantitative RT-PCR (H) ( $n = 3$ , technical replicates). Efficiency of skeletal myogenic differentiation was determined, as above, using G-Tool ( $n = 3$ ). Images are representative of three independent experiments.

(I) Y27632 improved the survival of MESP1-induced PDGFRA<sup>+</sup> cells by inhibiting apoptosis (Annexin V+ 7-AAD+/-).

Mean  $\pm$  SEM is shown in (A), (B), and (D)–(H). Scale bar represents 100  $\mu$ m in (C), (D), and (G). See also Figure S3 and Tables S1, S2, and S3.



ROCK signaling [Somlyo and Somlyo, 2000]) (Figures 3G and S3C). Y27632 was further shown to upregulate the expression of *Myod1* and *Myog* (Figure 3H). Moreover, similar to the protective role of ROCK inhibition for the survival of human pluripotent cells (Watanabe et al., 2007), Y28732 improved the survival of PDGFRA<sup>+</sup>-sorted cells cultured in serum-free conditions by inhibiting apoptosis (Figure 3I). In serum-containing (pro-cardiac) conditions, very few cells experienced apoptosis and Y27632 did not enhance the expression of cardiac genes (Figures S3D and S3E).

### PODXL and CDH4 Distinguish MESP1-Induced Early Cardiac and Skeletal Myogenic-Committed Cells, Respectively

The clear bifurcation between cardiac and skeletal myogenic differentiation and our ability to manipulate this CPM-like population by addition of cytokines makes it a powerful system for discovering early-acting factors within each lineage. We therefore performed RNA sequencing (RNA-seq) on four different CPM-derived populations: early cardiac (day 6, BMP4-treated for 24 hr), late cardiac (day 12, BMP4-treated for 7 days), early skeletal myogenic (day 6, untreated) and late skeletal myogenic (day 12, untreated) (Figures 4A, S4A, and S4B).

We reasoned that factors distinguishing cardiac versus skeletal myogenesis would be discovered at the earliest stage (i.e., day 6), and evaluated these genes for a membrane protein subset (Figures 4B and 4C). We further interrogated this list by antibody screening to identify potential early lineage-specific markers. We found that PODXL (podocalyxin) marked a portion of BMP4-treated cells (Figure 4D, left column). PODXL<sup>+</sup> cells were enriched for cardiac transcripts (Figure 4E, left two panels), and generated cardiac progenitors abundantly (Figure 4F, left panel). On the other hand, CDH4 (cadherin-4) labeled the majority of untreated cells, and its expression was substantially reduced by BMP4 (Figure 4D, right column). Skeletal myogenic gene expression was enriched in the CDH4<sup>+</sup> population (Figure 4E, right two panels), and skeletal myogenic progenitors could only be generated from these cells (Figure 4F, right panel). Interestingly, *Podxl* and *Cdh4* expression were downregulated in PODXL<sup>+</sup>- and CDH4<sup>+</sup>-sorted populations over time (Figure S4C), suggesting that both markers transiently label progenitors. In a conventional serum-throughout protocol, cardiac genes were also primarily expressed in the PODXL<sup>+</sup> fraction (Figure S4D). Our results therefore identify PODXL and CDH4 as surface markers to distinguish the earliest CPM-derived cardiac and skeletal myogenic-committed cells, respectively.

CPM is the only embryonic population with dual cardiac/skeletal myogenic potential, and little is known about

its regulation. There are some major obstacles hindering its study in an in vivo or ex vivo setting: the embryo at this early stage of development is very small, CPM is morphologically indistinguishable from other head mesoderm domains (Tzahor, 2015), and currently there is no known specific marker for its prospective isolation. An in vitro model of this bipotent tissue is of significant value in addressing these problems.

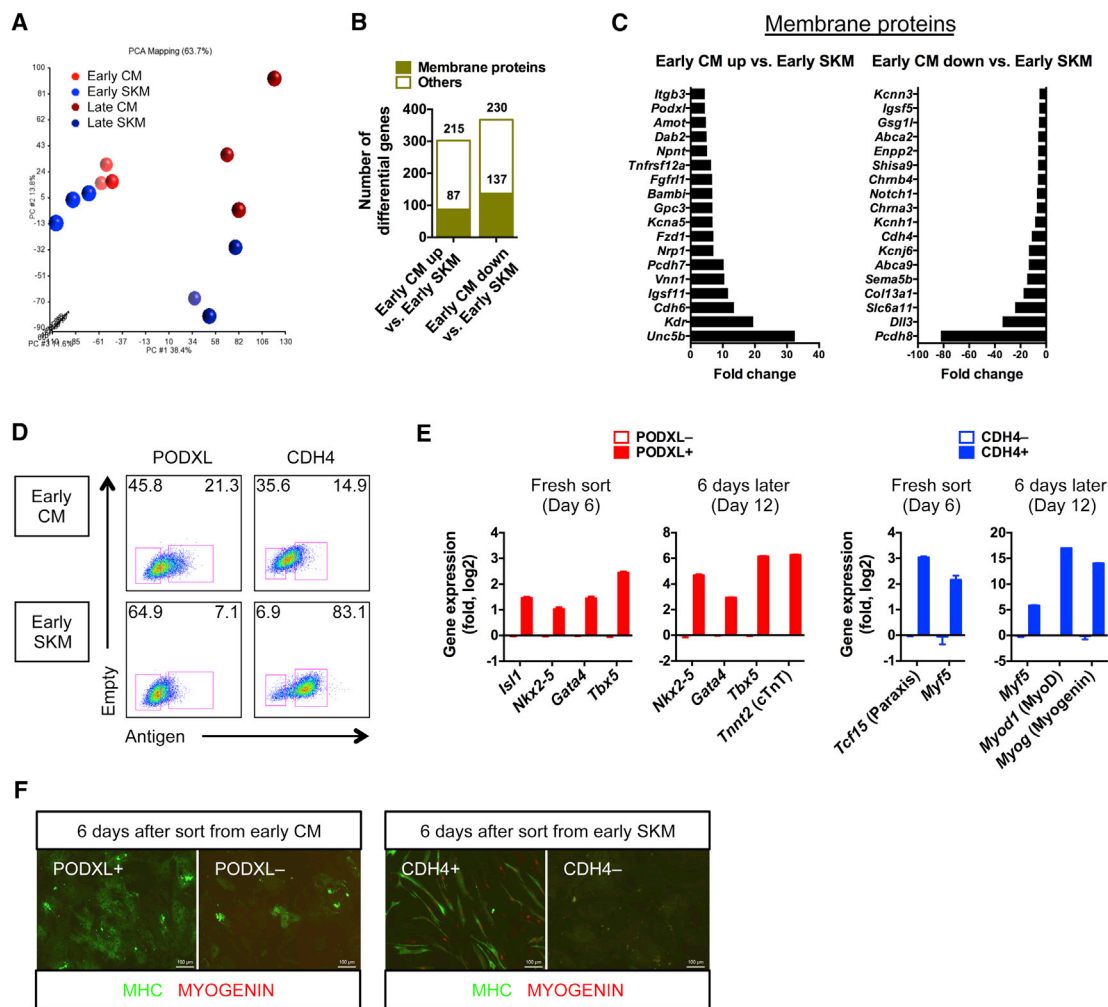
Under serum-free conditions in which ES cells do not differentiate into mesoderm, MESP1 is sufficient to drive mesoderm differentiation (Chan et al., 2013). We show here that this serum-free MESP1-induced mesoderm comprises a PDGFRA<sup>+</sup> progenitor population that functionally resembles the CPM, i.e., these cells can further differentiate into either cardiac or skeletal myogenic cells, or in some cases both simultaneously.

### In Vitro MESP1-Induced Mesoderm Allows Investigation of the Bifurcation of Early Cardiac and Skeletal Myogenic Progenitors

Using our in vitro CPM-modeling system, we have identified signaling pathways that enhanced cardiac or skeletal myogenic differentiation. We show that cardiac versus skeletal myogenesis of CPM is governed by signaling through an antagonistic TGFβ1/activin A-BMP2/4 axis, i.e., cardiogenesis is driven by both inhibition of TGFβ1/activin A signaling and stimulation of BMP2/4 signaling. TGFβ-BMP antagonism has been demonstrated in the differentiation of other musculoskeletal lineages, including bone (Spinel-la-Jaegle et al., 2001) and cartilage (Keller et al., 2011). Since the developing cranial anlage is surrounded with TGFβ and BMP signaling modulators (Bothe et al., 2011), this antagonistic TGFβ-BMP axis is likely relevant to the fates of other head mesoderm-derived lineages. We have also demonstrated that skeletal myogenesis is sensitive to Rho kinase signaling. These data validate the in vitro model for determining pathways involved in cardiac and skeletal myogenic differentiation.

The use of lineage-specific surface markers is fundamentally essential to distinguish and prospectively isolate lineage-primed/committed cells. KDR and PDGFRA have been extensively utilized to identify lateral plate mesoderm (leading to blood) and paraxial mesoderm (leading to trunk/limb skeletal muscles), respectively (Sakurai et al., 2006). Selection of the cardiac versus skeletal myogenic fate is rarely studied (Magli et al., 2014), and currently there are no surface markers to distinguish these two lineages from their common progenitor pool. Using the current in vitro system, we have identified and subsequently validated PODXL and CDH4 as surface markers for early CPM-derived lineage-restricted derivative progenitors.

PODXL was recently reported to mark early hematopoietic progenitors from another bipotent mesodermal progenitor



**Figure 4. PODXL and CDH4 Distinguish MESP1-Induced Early Cardiac and Skeletal Myogenic Progenitors, Respectively**

(A) Principal-component analysis of RNA-seq data generated from early (day 6) versus late (day 12) cardiac (CM) versus skeletal myogenic (SKM) populations (n = 3 independent experiments).

(B and C) RNA-seq analysis of early cardiac versus skeletal myogenic populations revealed a total of 669 genes whose expression was significantly altered during the early fate-specification process (B), wherein existed a membrane proteins subset (C).

(D) PODXL (podocalyxin) marked a portion of early cardiac progenitors (left column), and CDH4 (cadherin-4) marked most early skeletal myogenic progenitors (right column). FACS analysis was performed on MESP1-induced day-6 early cardiac (BMP4-treated, top row) and skeletal myogenic (untreated, bottom row) cells.

(E) PODXL+ sorted cells were highly enriched for cardiac transcripts (left two panels), and similarly, skeletal myogenic transcripts were abundantly found in CDH4+ sorted cells (right two panels) (n = 3, technical replicates). Mean ± SEM is shown.

(F) Immunostaining of day-12 cells previously sorted on day 6 based on PODXL and CDH4 expression. In BMP4-treated cells, cardiac progenitors were enriched in the PODXL+ fraction (left); whereas in untreated cells, skeletal myogenic progenitors were only observed in the CDH4+ fraction (right). Images are representative of three independent experiments. Scale bar represents 100 μm.

See also [Figure S4](#) and [Tables S1](#) and [S2](#).

cell, the hemangioblast (Zhang et al., 2014). Interestingly, early cardiogenic genes were enriched in both the KDR+ PODXL+ and KDR+ PODXL- populations. Surprisingly, in that study efficient cardiac differentiation was only observed from PODXL- cells. This later result may be due to the transient nature of podocalyxin expression, but may also reflect

differences between the two sequential waves of cardiac development, i.e., first heart field cells (previous report) and second heart field cells (current study).

The current study demonstrates the development of bipotent PDGFRA+ cardiac/skeletal myogenic progenitors from MESP1+ mesoderm, which molecularly and



functionally mimics the CPM of early embryogenesis. As such, this *in vitro* system represents a tractable model in which to study this developmentally important population.

## EXPERIMENTAL PROCEDURES

The CPM differentiation protocol is illustrated in Figure 1C. RNA-seq datasets can be accessed on GEO: GSE74682. Details of the following are included in the Supplemental Experimental Procedures: generation of MESP1-inducible ES cells, cell culture and differentiation, clonal analysis of single cells, gene expression and RNA-seq analysis, immunostaining, flow cytometry and apoptosis analysis, and chemical screening.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.12.003>.

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