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Heterogeneity of Mesp1+ mesoderm revealed by single-cell RNA-seq

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Abstract

Mesp1 is a transcription factor that promotes differentiation of pluripotent cells into different mesoderm lineages including hematopoietic, cardiac and skeletal myogenic. This occurs via at least two transient cell populations: a common hematopoietic/cardiac progenitor population and a common cardiac/skeletal myogenic progenitor population. It is not established whether Mesp1-induced mesoderm cells are intrinsically heterogeneous, or are simply capable of multiple lineage decisions. In the current study, we applied single-cell RNA-seq to analyze Mesp1+ mesoderm. Initial whole transcriptome analysis showed a surprising homogeneity among Mesp1-induced mesoderm cells. However, this apparent global homogeneity masked an intrinsic heterogeneity revealed by interrogating a panel of early mesoderm patterning factors. This approach enabled discovery of subpopulations primed for hematopoietic or cardiac development. These studies demonstrate the heterogeneic nature of Mesp1+ mesoderm.

Keywords

Mesp1; mesoderm patterning; cardiac development; hematopoiesis; single-cell RNA-seq

Introduction

Mesp1 is a transcription factor transiently expressed along the primitive streak as early as E6.5 during early embryogenesis [1]. Mesp1+ cells prominently contribute to heart formation [2] and its pro-cardiac effect has been well described [3–7]. Using a doxycycline (Dox)-inducible Mesp1 embryonic stem (ES) cell system, we observed that, depending on the culture environment, transient Mesp1 induction can give rise to cells of other lineages, including hematopoietic and skeletal muscle cells [8]. Interestingly, pro-skeletal muscle

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conditions appear to involve the differentiation of cardiopharyngeal mesoderm [9], i.e., a common cardiac/skeletal myogenic population [9–14]. This diversity suggests that different lineage-primed mesoderm subpopulations may simultaneously be present within the Mesp1+ domain. In fact, lineage-tracing analyses revealed that besides the heart, Mesp1+ cells also contribute to hematopoietic and skeletal myogenic development [8, 15]. Nevertheless, direct evidence for the presence of multiple distinct mesoderm subpopulations (e.g., hematopoietic and cardiac) upon Mesp1 activation is still lacking.

Single-cell RNA-seq has recently been advanced as a powerful tool to establish different cellular subgroups within an apparently homogeneous population [16]. For example, this strategy allowed the discovery of dendritic cell subsets with differential responses to an identical immune insult [17]. Therefore, single-cell RNA-seq stands out as an attractive approach to study the consequences of Mesp1 induction in ES cell derivatives. In the current study, we report a protocol in which hematopoietic and cardiac differentiation are simultaneously enhanced by Mesp1 induction. Based on this method, we employed single-cell RNA-seq to successfully identify several subpopulations within Mesp1+ mesoderm, including subsets primed for hematopoietic or cardiac development.

Materials and Methods

Generation of doxycycline-inducible MESP1 mouse ES cell line

The Dox-inducible MESP1 mouse ES cell line was engineered using the inducible cassette exchange strategy as described previously [8, 9, 18].

ES cell culture and differentiation

ES cells were cultured in maintenance medium on irradiated mouse embryonic fibroblasts (MEF) at 37 ºC in 5% CO_2. The maintenance medium was composed of: Knock-Out Dulbecco's Minimum Essential Medium (DMEM) (Life Technologies, Grand Island, NY), 15% ES cells-qualified fetal bovine serum (ES-FBS) (Gemini Bio-Products, West Sacramento, CA), 1% non-essential amino acids (NEAA) (Life Technologies), 1% penicillin/streptomycin (P/S) (Life Technologies), 2 mM Glutamax (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO) and 500 U/ml leukemia inhibitory factor (Millipore, Temecula, CA).

To initiate differentiation (day 0), MEFs were first depleted by plating dissociated ES cells on a tissue culture flask for 30–60 min. Single ES cells were then cultured in differentiation medium at 500,000 cells per 10 ml in non-adherent Petri dishes on an orbital shaker (80 rpm) at 37 ºC in 5% CO_2 to form embryoid bodies (EBs). The differentiation was composed of: Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies), 15% ES-FBS, 1% NEAA, 1% P/S, 2 mM Glutamax, 450 μM monothiolglyerol (Sigma), 200 μg/ml holo-transferrin (Life Technologies) and 50 μg/ml ascorbic acid (Sigma). To induce Mesp1 expression, doxycycline (500 ng/ml, Sigma) was added into the medium on day 2.25 and removed on day 3.25.

Flow cytometry analysis

EBs were collected on day 4, 6 and 8 for flow cytometry analysis. For surface marker staining, EBs were trypsinized and incubated with antibodies for 30 min on ice. Propidium iodide (PI) (1 μg/ml, Sigma) was added to differentiate between live and dead cells. Only live cells (PI−) were counted. For intracellular staining, dissociated cells were first fixed with 1% paraformaldehyde for 15 min at room temperature and permeabilized with ice-cold 90% methanol at −20 ºC overnight. Antibody staining was performed on the next day as described above except that PI was not added. Fluorescence activated cell sorting (FACS) were performed using a BD FACSAriaII (BD Biosciences, San Diego, CA) and data were analyzed using FlowJo (Tree Star, Ashland, OR). Antibodies used: Flk-1 (clone Avas12a1, BD Biosciences, San Diego, CA), PDGFRα (clone APA5, BD Biosciences), c-Kit (clone 2B8, eBioscience, San Diego, CA), CD41 (clone eBioMWReg30, eBioscience), and cardiac troponin T (clone CT3, Developmental Studies Hybridoma Bank, Iowa City, IA). The cardiac troponin T antibody was developed under the auspices of the NICHD and maintained by the University of Iowa.

Single-cell capture, RNA extraction and library creation

Total live cells (PI−) from day 4 Mesp1-induced EB cells were sorted by FACS. Sorted cells were then loaded into a medium-cell (10–17 μm) Fluidigm C1 mRNA Seq integrated microfluidic circuit for single-cell capture (Fluidigm, San Francisco, CA). Light microscopy verified an effective capture rate of 97.9% (out of 96 wells, 94 wells captured single cells and 2 wells captured 2 cells). RNA extraction, reverse transcription and cDNA amplification were performed on chip according to manufacturer's manual. Subsequently, library creation and RNA-seq were performed on 48 randomly selected single cells and the parent bulk population (10,000 cells).

Single-cell RNA-seq analysis

Sequencing was performed using an Illumina HiSeq2500. Reads were processed with Tophat 0.7 and Cufflinks 2.2.1.0 for transcriptome mapping and alignment [19, 20]. Further analyses, including gene expression correlation plots, hierarchical clustering, principal component analysis and differential genes determination were performed using R scripts (www.r-project.org) and Singular Analysis Toolset 3.5 (Fludigim) using default parameters.

Statistical analysis

Data are expressed as mean ± SEM. Student's t-tests or one-way analysis of variance (ANOVA) with Tukey post-hoc tests were performed for comparison between two groups or among three or more groups, respectively. Statistical significance was set as $p<0.05$.

Results

Mesp1 induction in a defined window promotes both hematopoietic and cardiac differentiation

Our previous work showed that Mesp1 induction at different stages of embryoid body (EB) differentiation produces opposite outcomes: an early 24 hr pulse generates hematopoietic

progenitors whereas a 24 hr-later 24 hr pulse generates cardiac progenitors [8]. To study potential heterogeneity of Mesp1+ mesoderm, we sought a time window in which both hematopoietic- and cardiac-primed mesoderm cells were produced simultaneously upon Mesp1 induction (Fig. 1A). We discovered that Mesp1 induction from day 2.25–3.25 promoted mesoderm patterning (Flk-1+ and/or PDGFRα+), as well as both hematopoietic (c-Kit+/− CD41+) and cardiac differentiation (cTnT+) (Fig. 1B–D). The enhanced hematopoietic and cardiac outcomes indicated that this transient pulse of Mesp1 promoted both hematopoietic- and cardiac-primed mesoderm cells. These phenotypic assays do not discriminate whether this population is homogeneous and bipotent, or intrinsically heterogeneous. Therefore, we employed single-cell RNA-seq analysis on cells arising immediately after the day 2.25–3.25 induction window.

Single-cell RNA-seq analysis reveals heterogeneity of Mesp1+ mesoderm cells

Day 4 Mesp1-induced mesoderm cells were first sorted by FACS to purify live cells and then loaded into the Fluidigm C1 microfluidic system for single-cell capture (Fig. 2A). We did not observe any preferences for particular cell sizes due to well location (i.e., bigger cells being captured first) as there was no correlation between RNA levels and well locations (Fig. 2B). RNA-seq was subsequently performed on 48 randomly selected cells. We observed an average concordant pair alignment rate of 82% with 6089 genes (FPKM > 1) being detected (Fig. 2C) – in concordance with reported data [21]. To determine the validity of the sampling of single cells, we compared the overall gene expression of individual cells and the parent bulk population of 10,000 cells. Initial analyses indicated that individual cells showed a good correlation with the bulk population, as well as among each other (Fig. 2D), suggesting superficially at least that the population is homogeneous.

The correlation methods used above are based on whole transcriptome expression. We reasoned that the effect of those key genes that regulate hematopoietic or cardiac specification, i.e., master regulators [22], might be masked by the vast majority of non-regulatory genes, making it difficult to detect heterogeneity. To investigate such a masking effect, we performed hierarchical clustering using a panel containing 13 major hematopoietic or cardiac regulatory factors. This analysis allowed us to appreciate 6 subpopulations (Fig. 2E). Principal component analysis (PCA) further revealed the degree to which hematopoietic and cardiac factors segregated these subpopulations (Fig. 2F). This biologically-informed single cell analysis thereby shows that Mesp1+ mesoderm is actually heterogeneous.

Progenitors at different stages of differentiation are present within Mesp1+ mesoderm

To understand the heterogeneity of Mesp1+ mesoderm, we compared the expression of the hematopoietic/cardiac panel genes among the 6 subpopulations (Fig. 3A–C). Functional heterogeneity would predict subsets enriched for one or the other, as opposed to random clustering, in which these 13 key regulatory factors would show no particular segregation. As predicted for functional heterogeneity, we observed a hematopoietic-primed subset and a cardiac-primed subset. Pro-hematopoietic factors such as *Etv2*, *Gata2*, *Tal1* (SCL) and *Lmo2* were enriched in Group F. In contrast, pro-cardiac factors including *Isl1*, *Mef2c*, *Tbx5* and *Myocd* (Myocardin) were predominantly found in Group E. We also observed less lineage-

committed subsets which appeared to lean towards either hematopoietic or cardiac specification. Group C had a similar level of *Etv2* (a master hematopoietic regulatory factor) to Group F, but the other pro-hematopoietic factors were only minimally expressed. On the other hand, whereas *Mesp1*, which promotes cardiac specification at this stage of differentiation (day 4) [8], and *Isl1*, a cardiac regulatory factor, were enriched in Group D, other pro-cardiac factors were not. Interestingly, Group B contained minimal levels of *Mesp1* and *Isl1* but relatively high levels of other pro-cardiac factors such as *Hand2*, *Meis1* and *Gata4*. That Group B and Group D expressed different sets of pro-cardiac regulatory factors may suggest the presence of two distinct cardiogenic programs, potentially related to the development of primary and secondary heart fields.

Group A had minimal expression of either hematopoietic or cardiac factors – these cells may be less differentiated or may have committed to other mesoderm lineages. Low expression of lineage-regulatory factors might also be due to a subpopulation of perturbed cells with abnormal transcriptional responses. The previous global correlations notwithstanding, we selectively examined the expression of several housekeeping genes (*Actb* (β-actin), *Gapdh* and *Eef2*) and found that they were expressed at similar levels among all groups (Fig. 3D), further arguing against this possibility.

Mesp1+ mesoderm subpopulations can be characterized by Kdr and Pdgfra expression

Flk-1 and PDGFRα are surface markers commonly used to define various mesoderm lineages including Flk-1+ putative lateral plate mesoderm leading to blood and PDGFRα+ putative cardiac/paraxial mesoderm leading to muscle [23–26]. It was therefore informative to examine their expression levels in our single cells and determine whether and how they might correlate with our population clustering strategy. Based on *Kdr* (which encodes Flk-1) and *Pdgfra* (which encodes PDGFRα) expression in the single cells, we derived 4 populations: *Kdr*+ *Pdgfra*+, *Kdr*− *Pdgfra*−, *Kdr*+ *Pdgfra*− and *Kdr*− *Pdgfra*+ (Fig. 4A). As expected, cells in the hematopoietic-primed Group F were primarily found in the *Kdr*+ *Pdgfra*− fraction and most of the cardiac-primed Group E cells were in the *Kdr*− *Pdgfra*+ fraction (Fig. 4B). Interestingly, more than half of Group C cells, which lean toward hematopoietic specification, were in the double-positive fraction, and Group D cells, which lean toward cardiac specification, were mainly in the *Kdr*− *Pdgfra*+ fraction (Fig. 4B).

Discussion

We have previously shown that Mesp1 promotes differentiation of various lineages via a common transient PDGFRα+ mesoderm population [8, 9]. The multipotent nature of these phenotypically similar cells suggests a potential heterogeneity among their transcriptome profiles. In the current study, we have provided evidence that Mesp1-induced mesoderm indeed contains various lineage-primed subpopulations including hematopoietic and cardiac.

Single-cell RNA-seq is an emerging tool for the study of cell population heterogeneity [16, 27]. To date, most reports have focused on well-defined cell populations such as cell lines [28] and adult tissues [17, 29–31], and studies on embryonic cell population are scarce [32, 33]. In the current study, we employed single-cell RNA-seq to examine the heterogeneity of Mesp1-induced mesoderm. The multipotent and developmentally transient nature of these

cells renders conventional analysis workflow, which assumes a stable non-plastic cell population, inappropriate. To circumvent this obstacle, we performed population clustering based on a curated list of major transcription factors regulating hematopoietic or cardiac specification, and discovered subpopulations primed for the development of these lineages. We further validated our clustering strategy by interrogating the expression levels of *Kdr* and *Pdgfra*, two widely used markers for distinguishing hematopoietic (*Kdr*+ *Pdgfra*−) and cardiac (*Kdr*− *Pdgfra*+) mesoderm [23–26]. As predicted, the putative hematopoietic subpopulation (Group F) is mainly *Kdr*+ *Pdgfra*−, whereas the putative cardiac subpopulation (Group E) is predominantly *Kdr*− *Pdgfra*+. Therefore, our clustering strategy is capable of distinguishing hematopoietic and cardiac fractions from Mesp1+ mesoderm and furthermore, supports the notion that hematopoietic and cardiac progenitors derive from Flk-1+ and PDGFRα+ cells respectively [23, 24].

In this report, we have demonstrated the presence of hematopoietic and cardiac lineages within Mesp1+ mesoderm by using single-cell RNA-seq technology. Our clustering strategy based on the expression of lineage regulatory factors is both simple to implement and readily amendable. Our approach would be applicable for analyzing the heterogeneity of other multipotent cell population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Dox	doxycycline
ES	embryonic stem (cell)
EB	embryoid body

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Highlights

- A defined Mesp1 window promotes both hematopoietic and cardiac differentiation
- Single-cell RNA-seq reveals heterogeneity of Mesp1+ mesoderm
- Mesp1+ mesoderm contains a hematopoietic-primed and a cardiac-primed subset

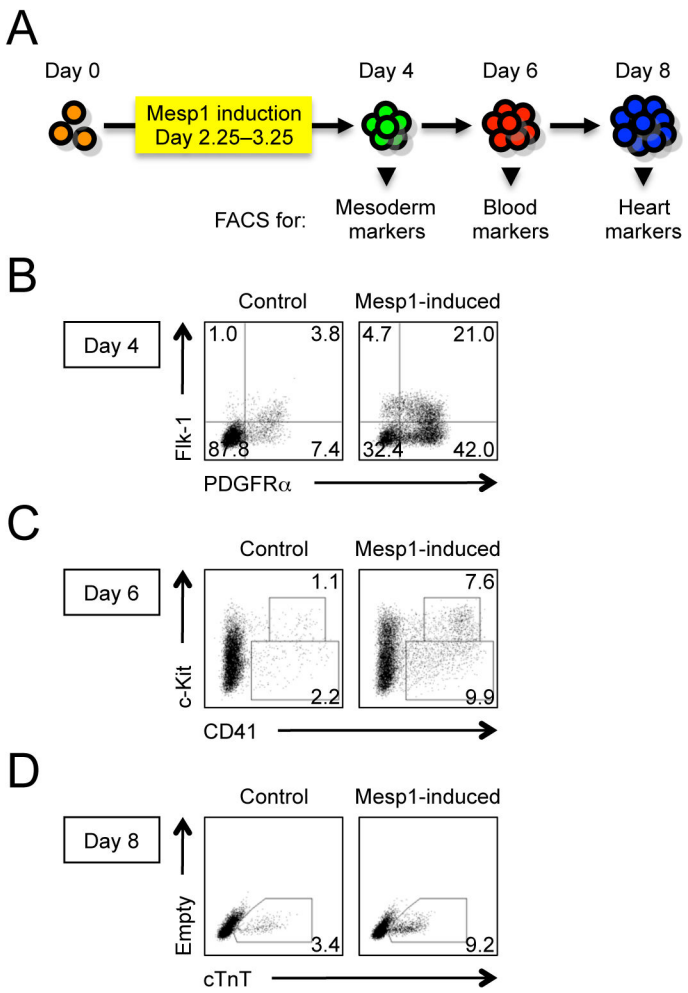

Figure 1. Transient induction of Mesp1 within a defined window promotes both hematopoietic and cardiac differentiation

(A) Scheme depicting the protocol used to evaluate the hematopoietic and cardiac outcomes of Mesp1 induction by Dox from day 2.25–3.25 on ES cell differentiation.

(B–D) FACS analysis showing that a defined transient pulse of Mesp1 promoted mesoderm patterning (Flk-1+ and/or PDGFRα+) by day 4 (B), hematopoietic differentiation (c-Kit+/− CD41+) by day 6 (C), and cardiac differentiation (cTnT+) by day 8.

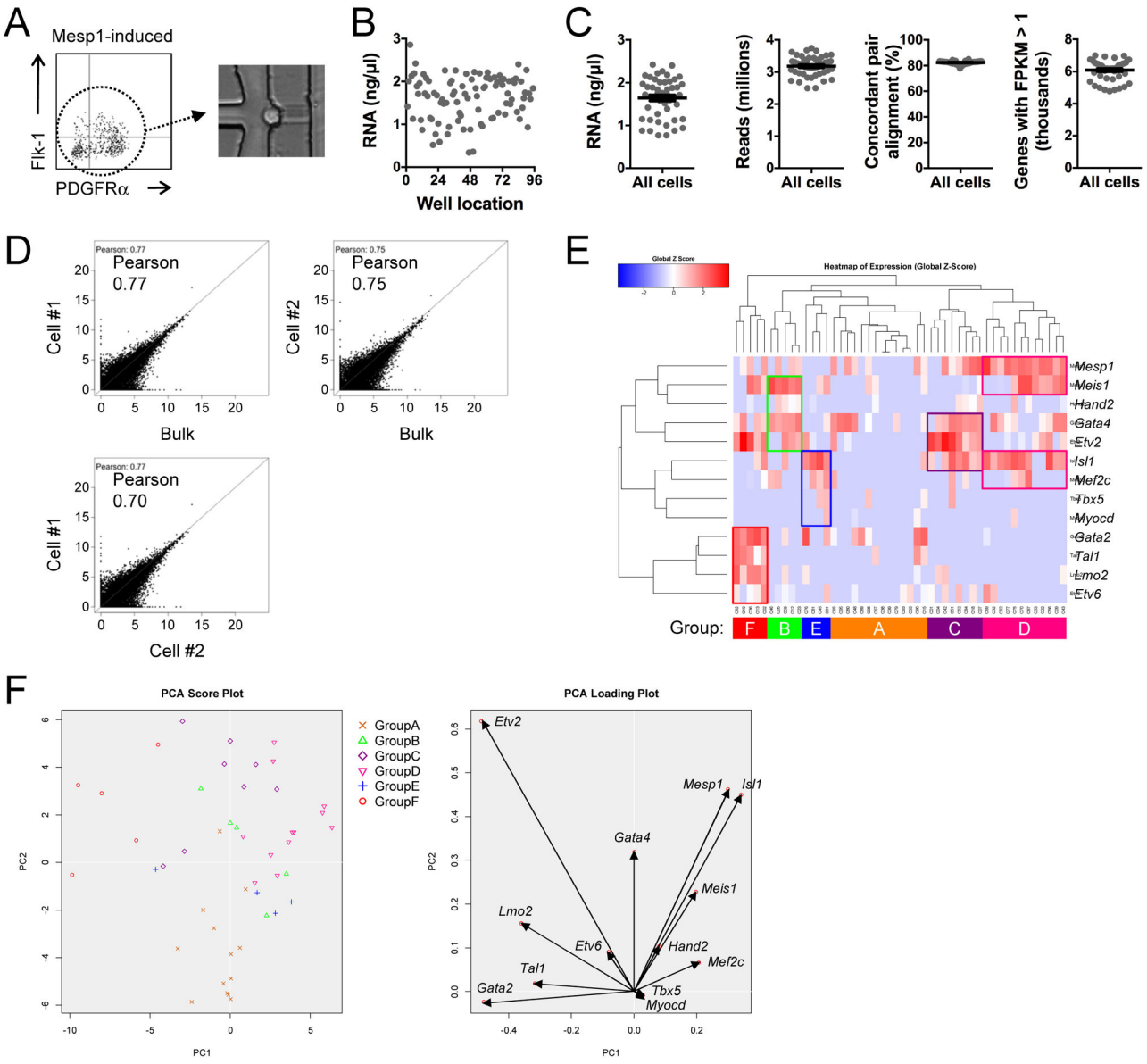

Figure 2. Heterogeneity of Mesp1+ mesoderm cells

(A) Day 4 Mesp1+ mesoderm cells were loaded into the Fluidigm C1 microfluidic system for single cell capture. Right panel shows a captured cell.

(B) Varying levels of RNA were observed in 94 single cells. Forty-eight single cells were randomly selected for RNA-seq.

(C) Quantification of RNA (far left), sequenced reads (near left), overall read alignment (middle), concordant pair alignment (near right) and genes with FPKM > 1 (far right) in 48 single cells. Mean ± SEM is shown.

(D) Representative correlation plots of the abundance of mapped genes (FPKM > 1) among individual cells and the bulk control. A good correlation was observed between individual cells and a bulk sample of 10,000 cells (top row), and also among individual cells (bottom row).

(E) Hierarchical clustering from a set of hematopoietic and cardiac genes revealed 6 distinct subpopulations (Group A–F) within Mesp1+ mesoderm cells.
(F) Principal component analysis (PCA): score plot (left) and loading plot (right).

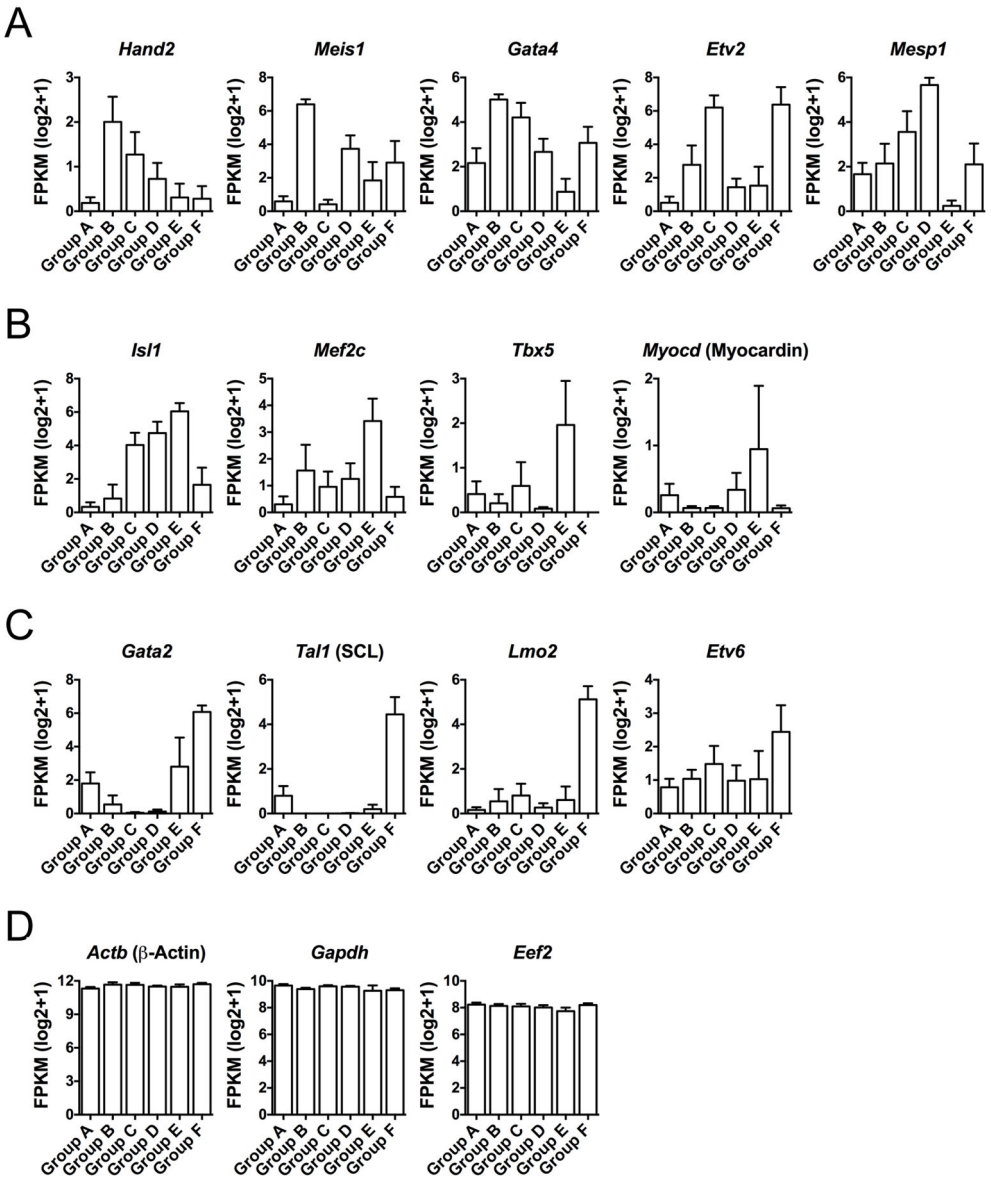

Figure 3. Mesp1+ mesoderm contains progenitors at different stages of differentiation

(A–D) FPKM values of early mesoderm genes (A), cardiac genes (B), hematopoietic genes (C) and housekeeping genes (D) among the 6 Mesp1+ mesoderm subpopulations. Mean ± SEM is shown.

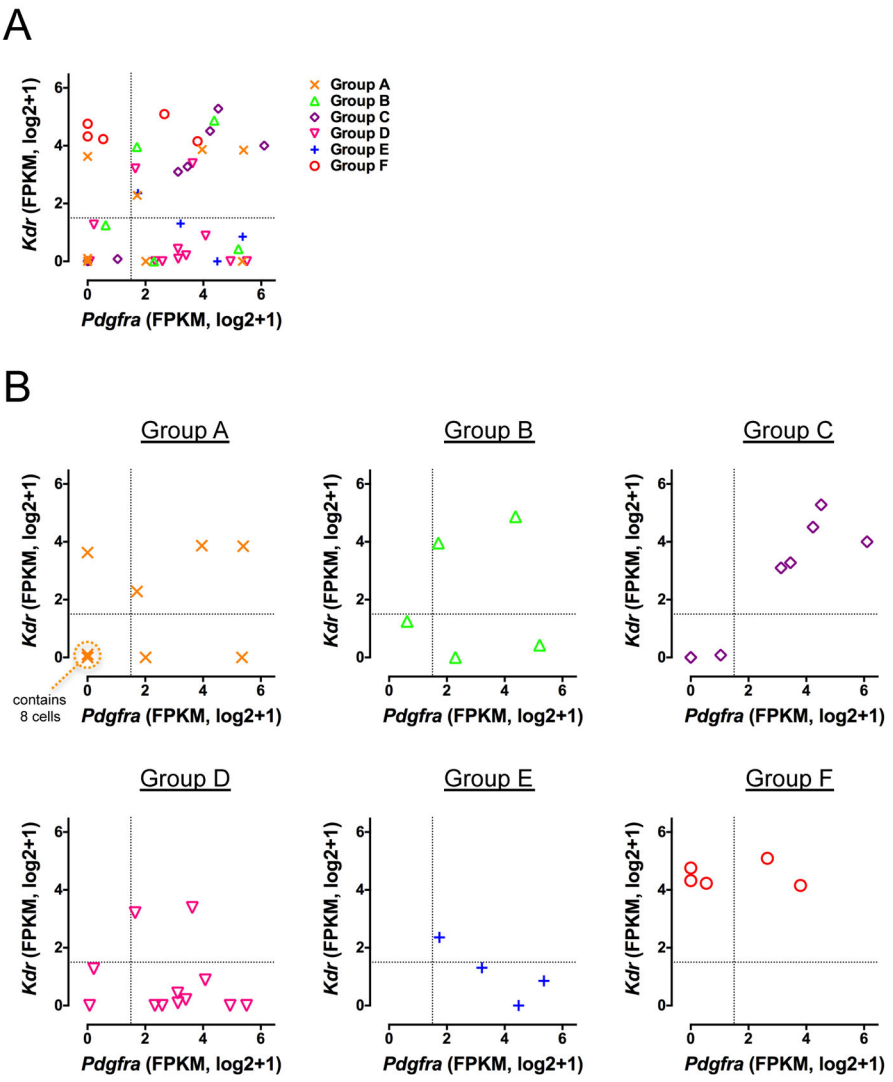

Figure 4. *Kdr* and *Pdgfra* expression in Mesp1+ mesoderm subpopulations

(A, B) Grouping of single Mesp1+ mesoderm cells based on *Kdr* and *Pdgfra* expression (*Kdr*− *Pdgfra*−, *Kdr*+ *Pdgfra*+, *Kdr*− *Pdgfra*+ and *Kdr*+ *Pdgfra*−): (A) all 48 single cells and (B) individual subpopulations.