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.

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1. 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 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 identify several subpopulations within Mesp1+ mesoderm, including subsets primed for hematopoietic or cardiac development.

2. Materials and methods

2.1. 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].

2.2. ES cell culture and differentiation

ES cells were cultured in maintenance medium on irradiated mouse embryonic fibroblasts (MEF) at 37 °C 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 cell-qualified fetal bovine serum (ES-FBS) (Gemini Bio-Products, West Sacramento, CA), 1% non-essential amino acids (NEAA) (Life Technologies), 1% penicillin/strepto-mycin (P/S) (Life Technologies), 2 mM Glutamax (Life Technolo-gies), 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 °C 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 monothioglycerol (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.

2.3. 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 °C 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.

2.4. 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 micro-scopy 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. Subse-quently, library creation and RNA-seq were performed on 48 randomly selected single cells and the parent bulk population (10,000 cells).

2.5. 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 tran-scriptome 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.

2.6. 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 per-formed for comparison between two groups or among three or more groups, respectively. Statistical significance was set as $p < 0.05$.

3. Results

3.1. 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 pulse generates hematopoietic progenitors whereas a 24 h-later 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 in-duction (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 he-matopoietic 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 intrin-sically heterogeneous. Therefore, we employed single-cell RNA-seq analysis on cells arising immediately after the day 2.25–3.25 in-duction window.

3.2. 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 tran-scriptome 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 clus-tering using a panel containing 13 major hematopoietic or cardiac regulatory factors. This analysis allowed us to appreciate 6 sub-populations (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+ meso-derm is actually heterogeneous.