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J Philip Creamer  
Washington University School of Medicine in St. Louis  
Stephanie A Luff  
Washington University School of Medicine in St. Louis  
Hao Yu  
Icahn School of Medicine Mount Sinai  
Christopher M Sturgeon  
Washington University School of Medicine in St. Louis

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CD1d expression demarcates CDX4+ hemogenic mesoderm with definitive hematopoietic potential

J. Philip Creamer a, Stephanie A. Luff a,b,c, Hao Yu b,c, Christopher M. Sturgeon a,b,c,*

a Department of Medicine, Division of Hematology, Washington University School of Medicine, St. Louis, MO, United States
b Department of Cell, Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY, United States
c Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai School of Medicine, New York, NY, United States

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ABSTRACT
To achieve efficient, reproducible differentiation of human pluripotent stem cells (hPSCs) towards specific hematopoietic cell-types, a comprehensive understanding of the necessary cell signaling and developmental trajectories involved is required. Previous studies have identified the mesodermal progenitors of extra-embryonic-like and intra-embryonic-like hemogenic endothelium (HE), via stage-specific WNT and ACTIVIN/NODAL, with GYPHA/GYPB (CD235a/b) expression serving as a positive selection marker for mesoderm harboring exclusively extra-embryonic-like hemogenic potential. However, a positive mesodermal cell-surface marker with exclusively intra-embryonic-like hemogenic potential has not been identified. Recently, we reported that early mesodermal expression of CDX4 critically regulates definitive HE specification, suggesting that CDX4 may act in a cell-autonomous manner during hematopoietic development. To identify CDX4+ mesoderm, we performed single cell (sc)RNAseq on hPSC-derived mesodermal cultures, revealing CDX4+ expressing mesodermal populations were uniquely enriched in the non-classical MHC-Class-1 receptor CD1D. Flow cytometry demonstrated approximately 60% of KDR+CD34-CD235a- mesoderm was CD1d+, and CDX4 was robustly enriched within CD1d+ mesoderm. Critically, only CD1d+ mesoderm harbored CD34+HOX4+ HE with multilineage erythroid-myeidoid-lymphoid potential. Thus, CDX4+CD1d+ expression within early mesoderm demarcates an early progenitor of HE. These insights may be used for further study of human hematopoietic development and improve hematopoietic differentiation conditions for regenerative medicine applications.

1. Introduction
A long-held goal of regenerative medicine has been the in vitro production of hematopoietic stem cells (HSCs) that can be used therapeutically and as a platform for the study of hematological disease. Despite recent advances in the field of directed differentiation of human pluripotent stem cells (hPSCs), this goal remains unrealized in the absence of transgene expression (Sugimura et al., 2017). This is due, in part, to our limited understanding of the overall complexity and heterogeneity of embryonic hematopoietic development, where multiple, spatiotemporally separated programs have been identified (Ditadi et al., 2017). Broadly, these can be separated into extra-embryonic, erythroid-myeloid programs and the intra-embryonic (definitive) multilineage programs, the latter of which gives rise to the HSC from hemogenic endothelium (HE) within the dorsal aorta (Gritz and Hirschi, 2016).

However, the mesodermal origin(s) of HE remains poorly defined. The hPSC differentiation system has identified several markers of early mesoderm with hemogenic potential, such as KDR (Kennedy et al., 2007), PDGFRα (Davis et al., 2008); and APLNR (Yu et al., 2012). However, these markers do not discriminate between other mesodermal lineages, nor do they define progenitors of the extra-embryonic-like and intra-embryonic-like hematopoietic programs. We have previously identified that mesodermal expression of GYPHA/GYPB (CD235a/b) demarcates a population within hPSC differentiation cultures that harbors exclusively extra-embryonic-like hematopoietic potential, and that the expression of CD235a/b on this nascent mesoderm is regulated by stage-specific WNT and ACTIVIN/NODAL signaling (Sturgeon et al., 2014). However, the identification of a cell surface antigen that positively identifies a mesodermal population with exclusively definitive hematopoietic potential, but not extra-embryonic-like potential, has

* Corresponding author at: Icahn School of Medicine at Mount Sinai, Black Family Stem Cell Institute, One Gustave L. Levy Place, Box 1496, New York, NY 10029, United States.
E-mail address: christopher.sturgeon@mssm.edu (C.M. Sturgeon).

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remained elusive. WNT-mediated expression of CDX1/2/4 has been correlated with specification of KDR+/CD235a- mesoderm harboring potential for CD34+/HOXA+ intra-embryonic-like cells with hematopoietic potential (Ng et al., 2016; Creamer et al., 2017; Jung et al., 2021). Thus, we aimed to use these CDX genes to identify definitive hematopoietic precursors within mesodermal hPSC cultures.

2. Materials and methods

2.1. hPSC maintenance and differentiations

The hPSC line H1 (WA01; WiCell) was cultured and differentiated as described previously (Sturgeon et al., 2014; Creamer et al., 2017; Kennedy et al., 2012; Ditadi and Sturgeon, 2016). Briefly, hPSCs were MEF-depleted by culturing on Matrigel (BD Biosciences) in hESC media for 24 hr. Embryoid bodies (EBs) were generated by treating hPSCs with trypsin-EDTA (0.05%) for 1 min. Cells were detached by scraping to form small aggregates (6–10 cells). EBs were resuspended in N2/B27-based media (Sturgeon et al., 2012) supplemented with L-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 4x10⁻⁴ M; Sigma), holo-transferrin (150 µg/ml), BMP-4 (10 ng/ml). After 24 hr, the media was supplemented with bFGF (5 ng/ml) and following 42 hr total, the media was changed using the same media above supplemented with CHIR99021 (3 µM) and SB43152 (6 µM) (Ng et al., 2016). Following 72 hrs of differentiation, EBs were dissociated with treatment of 0.25% Trypsin-EDTA (5 min), followed by FACS (BD Biosciences) isolation of KDR +/CD235a-CD1d+/mesoderm. FACS isolated cells were re-aggregated at 2.5x10⁴cells/ml and placed in StemPro-34 media (Gibco) supplemented with L-glutamine (2 mM), ascorbic acid (1 mM), mononitroglycerol (MTG, 4 x 10⁻⁴ M), holo-transferrin (150 µg/ml), VEGF (15 ng/ml), and bFGF (5 ng/ml). After 3 days, cultures were fed with an equal volume of StemPro-34 media supplemented as previously described, but with additional IL-6 (10 ng/ml), IGF-1 (25 ng/ml), IL-11 (5 ng/ml), SCF (50 ng/ml), and EPO (2 U/ml), and cultured for an additional 2 days.

2.2. Erythro-myeloid potential

Resultant CD34+/CD43- cells were isolated by FACS and then assessed for hematogenic potential as described (Ditadi et al., 2015). Briefly, cells were aggregated overnight at a density of 2 x 10⁶cells/ml in 96 well plates with StemPro-34 media, containing L-glutamine (2 mM), ascorbic acid (1 mM), mononitroglycerol (MTG, 4 x 10⁻⁴ M), holo-transferrin (150 µg/ml), TPO (30 ng/ml), IL-3 (30 ng/ml), SCF (100 ng/ml), IL-6 (10 ng/ml), IL-11 (5 ng/ml), IGF-1 (25 ng/ml), EPO (2 U/ml), VEGF (5 ng/ml), bFGF (5 ng/ml), BMP4 (10 ng/ml), Flt-3L (10 ng/ml), and SHH (20 ng/ml). Aggregates were then transferred onto Matrigel-coated plasticware cultured for an additional 8 days in the same media. All differentiation cultures were maintained in a 5% CO₂and 5% O₂environment. All recombinant factors are human and were purchased from R&D Systems (Minneapolis, MN). Analysis of hematopoietic colony forming cell potential via Methocult (StemCellTechnologies) was performed as described previously (Ditadi et al., 2015).

2.3. T-lymphoid assay

T-lymphoid potential was assessed using OP9-DL4 stroma (La Motte-Mohs et al., 2005; Schmitt et al., 2004) as described previously (Sturgeon et al., 2014; Kennedy et al., 2012; Ditadi et al., 2015). Briefly, 1 x 10⁵CD34+/CD43- cells were added to individual wells of a 24-well plate containing OP9-DL4 cells and cultured alpha-MEM supplemented with 20% FBS, SCF (30 ng/ml), IL-7 (5 ng/ml). After 5 days, cultures were maintained in 6-well plates of OP9-DL4 in the absence of SCF. Every four to five days co-cultures were transferred onto fresh OP9-DL4 cells by vigorous pipetting andpassaging through a 40 µm cell strainer. Cultures were assayed following 28 days for the presence of a CD45+CD56-CD4+CD8+ population. Cultures exhibiting at least 100 CD4+CD8+ cells were considered positive for T-lymphoid potential.

2.4. Single cell RNAseq analyses

Day 3 differentiation cultures were dissociated and immediately fixed with methanol as previously described (Alles et al., 2017). Briefly, EB’s were treated with trypsin-EDTA for 5 min, stopped with 5% FBS + IMDM, and spun at 300 x g for 5 min. After resuspending with PBS + 5% FBS and counting, cells were pelleted at 300 x g for 5 min at 4°C, the supernatant was removed manually, and the cell pellet resuspended in 2 volumes (200 µl) of ice-cold PBS. To avoid cell clumping, 8 volumes (800 µl) of methanol (grade p.a.; pre-chilled to ~20°C) were added dropwise, while gently vortexing the cell suspension (final concentration: 80% methanol in PBS). The methanol-fixed cells were kept on ice for a minimum of 15 min and then stored at ~80°C. For rehydration, cells were kept on ice, pelleted at 1000 x g, washed and resuspended in PBS + 0.01% BSA, passed through a 40-µm cell strainer, counted and diluted for library prep (1000 cells per µl). Libraries were prepared following the manufacturer’s instruction using the 10X Genomics Chromium Single Cell 3’ Library and Gel Bead Kit v2 (PN120237), Chromium Single Cell 3’ Chip kit v2 (PN-120236), and Chromium (7 Multiplex Kit (PN-120262). 17,000 cells were loaded into a well of the chip, capturing > 6000 cells. cDNA libraries were sequenced on an Illumina HiSeq 3000. Sequencing reads were processed using the Cell Ranger software pipeline (version 2.1.0). Using Seurat (Stuart et al., 2019) (version 3.9.9) implemented in R (version 4.0.3) for all steps described below, the dataset was filtered by removing genes expressed in <3 cells, and retaining cells with unique gene counts between 200 and 6000. The remaining UMI counts were normalized and transformed, and regression was performed to account for the percent of mitochondrial UMI counts. Principal component analysis was used to generate uniform manifold approximation and project (UMAP) plots and unsupervised clustering was performed using a resolution of 0.9, resulting in 12 cell clusters. One cluster was removed for excess mitochondrial gene contribution (median > 10%). Differential gene expression analysis was performed using the ‘FindAllMarkers’ function with a minimum expression threshold of 25%, 0.25 log2 fold change, and padj (Benjamini-Hochberg) < 0.01 to determine the cell identity of each cluster. The dataset is publicly available at the Gene Expression Omnibus (GEO) under the accession number: GSE139850.

2.5. RNA expression analysis

For qRT-PCR, total RNA was isolated with the RNeasy Mini Kit (Qiagen) using TRI Reagent (Sigma-Aldrich). For reverse transcription, cDNA was synthesized using the SuperScript III kit (Invitrogen). Real-time quantitative PCR was performed on a StepOnePlus thermocycler (Applied Biosystems), using Power Green SYBR mix (Invitrogen). Gene expression values were calculated using the ΔCt method against the endogenous control (ACTB). Primer sequences are:

| Gene   | Forward primer | Reverse primer |
|--------|----------------|----------------|
| CDX1   | TGAACCGAGGTAGGATCAG | CTGTCATCCCGGGTCTT |
| CDX2   | CGAAGGTTTACCTGGCGGA | GGTGCTCGGATCTTGGTCA |
| CDX4   | CCTTTCGGAGACAGCTGGA | CACGGAGCCTCACTATTC |
| CDX7   | AGGAGACATCAAGGCGTTA | HOX9A9 | CGAAGGACGGTCAAGCTGATC |
| CDX9   | TGGCTACCTCAGCTTTCGTC | RUNX1 | GGTCGACATCCATGCCAAGAGTTAAG |

2.6. Flow cytometry and cell sorting

Antibodies used include KDR-PE/KDR-PE-Cy7 (clone 89106, R&D Systems). CD34-APC (clone 8G12), CD34- PE-Cy7 (clone 4H11), CD43-PE (clone 8G12), ascorbic acid (1 mM), monothioglycerol (MTG, 4x10⁻⁴ M; Sigma), holo-transferrin (150 µg/ml), BMP-4 (10 ng/ml). The remaining UMI counts were normalized and transformed, and regression was performed to account for the percent of mitochondrial UMI counts. Principal component analysis was used to generate uniform manifold approximation and project (UMAP) plots and unsupervised clustering was performed using a resolution of 0.9, resulting in 12 cell clusters. One cluster was removed for excess mitochondrial gene contribution (median > 10%). Differential gene expression analysis was performed using the ‘FindAllMarkers’ function with a minimum expression threshold of 25%, 0.25 log2 fold change, and padj (Benjamini-Hochberg) < 0.01 to determine the cell identity of each cluster. The dataset is publicly available at the Gene Expression Omnibus (GEO) under the accession number: GSE139850.
As we previously identified a critical role for mesodermal CDX4 expression during the hematopoietic differentiation of hPSCs (Creamer et al., 2017), we first sought to characterize the expression of CDX1/2/4 at a single cell level within hPSC-derived mesoderm under WNT-dependent intra-embryonic-like culture conditions. To facilitate a better understanding of the overall heterogeneity as well as CDX expressing cells within nascent hPSC-derived mesoderm, we leveraged single cell (sc)RNAseq. We assayed cultures on day 3 of differentiation, which contain KDR + CD235a- mesoderm that expresses CDX genes in a WNT-dependent manner (Creamer et al., 2017) (Fig. 1A). These cultures are at a distinct developmental stage that precedes hematopoietic specification (Sturgeon et al., 2014; Ng et al., 2016; Kitajima et al., 2016), which we refer to as “hemogenic mesoderm”. Following standard data processing and UMAP generation, unsupervised clustering was performed using Seurat (Stuart et al., 2019) which identified 11 distinct clusters (Fig. 1B). To resolve the identity of many of these clusters, we identified differentially enriched genes (DEGs) within each cluster, revealing a striking degree of heterogeneity within the cultures (Supplementary Table 1). Distinct transcriptional identities for many developmental intermediates could be identified in each cluster, including residual pluripotent stem cells (SOX2, NODAL), ectoderm (TPA28, KRIT, NRN1), neuroectoderm (NRN1), primordial germ cell (NANOS3, KIT), early mesoderm (TBXT, HES7), endoderm (HNFP1, FOXA2), visceral endoderm (TTR), mesoderm (MESP1, TBX4, OSR1), and endothelial progenitors / endothelium (PLAT, TEK, CDHS, GATA2) (Fig. 1C). As the “Early Mesoderm”, “Mesoderm A” and “Mesoderm B” (clusters 5–9 respectively) all expressed markers associated with early mesoderm (KDR, APLNR, PDGFRa), but preceded hematodentinal specification (TEK, CDHS, GATA2; Fig. 1C) (Sturgeon et al., 2014; Creamer et al., 2017), we focused on these cells as candidate hemogenic mesodermal populations.

We and others (Ng et al., 2016; Creamer et al., 2017; Jung et al., 2021; Davidson et al., 2003; Wang et al., 2008) have previously demonstrated that early expression of CDX1/2/4 correlates with definitive hematopoietic specification, and that CDX4 acts as critical regulator of HE development from its mesodermal precursor (Creamer et al., 2017). We therefore hypothesized that CDX4(+) clusters may contain these precursors to definitive HE, and could be used to identify unique cell surface antigens for their identification in vitro. Differential gene expression testing performed on each cluster compared to all remaining clusters revealed that CDX4 was positively enriched only within the “Early Mesoderm” (cluster 5) and “Mesoderm A” (cluster 8), but not within “Mesoderm B” (Supplementary Table 1). This led us to designate “Early Mesoderm” and “Mesoderm A” as the “CDX4+” clusters within the dataset. While “Mesoderm B” did not have significantly enriched CDX4 in comparison to the rest of the dataset, low level CDX4 expression was detectable, leading us to designate this cluster as “CDX4-”, and the remaining clusters, comprised of all other cell types, as “CDX4-” (Fig. 1D/E). Interestingly, when differential gene analysis was performed across these newly defined groups, CD1D, a non-canonical MHC receptor found on antigen presenting cells (Beckman, 1994), was found to be enriched in the CDX4+ clusters and correlated with high CDX1/2/4 expression (Supplementary Table 2, Fig. 1F).

Flow cytometric analysis of these differentiation cultures on day 3 following CHIR90021 and SB43152 treatment, 61.47 +/- 3.121% (SEM, n = 6) of KDR + CD34+CD235a- mesoderm was CD1D+ (Fig. 2A). While FACS-isolated CD1D+ cells were 8-fold enriched and 20-fold enriched respectively in CDX1 and CDX4 expression (Fig. 2B), there was a non-statistically significant ~2-fold enrichment of CDX2 (p = 0.0545). This agrees with the observation in the scRNAseq dataset that the CDX4- and CDX4+ populations also expressed CDX2 but lacked any CD1D expression (Fig. 1E/F). To determine the hematopoietic potential of each mesodermal population, KDR+CD235a-CD1Dneg/hi cells were isolated by FACS and cultured an additional 5 days under hematopoietic promoting conditions (Sturgeon et al., 2014). Interestingly, each population gave rise to a CD34+CD43- population at similar frequencies (Fig. 2Aii). Gene expression analysis of isolated CD34+ endothelium from each culture revealed that the CD1D+ derived endothelium was significantly enriched for HOXAT/9 and RUNX1 expression in comparison to those derived from CD1Dneg mesoderm (Fig. 2C), suggesting that hemogenic potential may be restricted to the CD1D-derived CD34+ cells. We therefore isolated each by FACS to assess for the presence of functional HE (Ditadi et al., 2015). After culturing as a monolayer for an additional 9 days under endothelial-to-hematopoietic transition (EHT) promoting conditions, the CD1Dneg derived CD34+ cells exhibited no signs of multilineage hematopoietic potential. However, the CD1D+ -derived CD34+ cells exhibited robust hematopoietic potential, with many round non-adherent hematopoietic cells observed in the culture (Fig. 2D). These cells were confirmed to be hematopoietic progenitors, as when we transferred this population to methylcellulose, many erythro-myeloid CFUs were detected (Fig. 2E).

Having observed that nearly all definitive erythro-myeloid lineage potential was contained within the KDR+CD235a-CD1D+ mesoderm, we next wanted to assess the presence of T-lymphoid potential, as multilineage erythro-myeloid and T-lymphoid potential are a defining characteristic of hPSC-derived definitive hematopoiesis (Kennedy et al., 2012). After isolation of KDR+CD235a-CD1Dneg/hi+ cells on day 3, CD34+CD43- progenitors were further FACS purified on day 8 and co-cultured with OP9-DL4 stroma (La Morte-Mohs et al., 2005; Schmitt et al., 2004) under T-cell promoting conditions (Sturgeon et al., 2014; Kennedy et al., 2012; Ditadi et al., 2015). After 22 days, flow cytometric analysis confirmed that only CD34+ cells derived from CD1D+ mesoderm were able to give rise to CD45+CD56-CD4+CD8- T-cells in vitro (Fig. 2Aii). Collectively, these results demonstrate that CD1D+ cells demarcates nascent hemogenic mesoderm with definitive hematopoietic potential.

In summary, single cell transcriptomics of early stage hPSC differentiation cultures has revealed a subpopulation of mesoderm, preceding hematopoietic specification, that is highly enriched for both CDX4 and CD1D. Subsequent functional in vitro studies demonstrate that CD1D is a marker for CDX4+ hemogenic mesoderm with multilineage definitive erythroid, myeloid, and lymphoid potential. While this does not demonstrate a cell-autonomous role for CDX4 in the development of the definitive hematopoietic program, the strong correlation between CD1D and CDX4 expression, coupled with our previous studies demonstrating that CDX4 regulates definitive hematopoietic development within
Fig. 2. Phenotypic and functional characterization of CD1d expressing cells within day 3 mesoderm A.(i) Representative flow cytometry of day 3 differentiation cultures for KDR, CD235a, CD34, and CD1d. KDR+CD235a− definitive hemogenic mesoderm was then assessed for the expression of CD1d (n = 6). (ii) Representative flow cytometry plots of cultures as in (i), reaggregated for an additional 5 days (n = 6). Each resultant culture was assessed for CD34 and CD43 expression. CD34+CD43− cells were isolated by FACS. (iii) Representative flow cytometry assay for T cell potential of each culture as in (ii). Following 22 days of coculture, cells were harvested for flow cytometric analysis of CD45−CD56− population for CD4 and CD8 expression, n = 3. B. Fold change in expression of CDX genes via qPCR in KDR+CD235a−CD1d+/− FACS isolated cells on day 3 of differentiation. n = 3, +/- SEM, * p < 0.05, *** p < 0.001 via students t test. C. Normalized expression levels of HOXA7, HOXA9, and RUNX1 genes, as determined by qRT-PCR, within CD34+CD43− cells derived from KDR+CD1d+/− or KDR+CD235a+ mesoderm. n = 6 (n = 5 for RUNX1), +/- SEM, * p < 0.05, ** p < 0.01, and **** p < 0.0001 via Turkey’s Multiple comparisons test. D. Representative micrographs of cultures of isolated CD34+CD43− cells, as in Aii, cultured as a monolayer for another 9 days in endothelial to hematopoietic transition (EHT) promoting media, 100X magnification, scale bar = 100 µm. E. Erythroid-myeloid potential of EHT cultures as in C. Cultures were harvested and placed into hematopoietic methylcellulose media for colony forming assays. The numbers of colonies were then assessed after 10–12 days and the numbers were counted of burst forming units erythroid (BFU-E) and colony forming units (CFU) of erythroid (E), granulocyte (G), myeloid (M), and mixed granulocyte/myeloid (GM). n = 6, +/- SEM, **** p < 0.0001 2-way ANOVA.
mesoderm, is consistent with the hypothesis that CD1d identifies CDX4+ hemogenic mesoderm. Notably, not all CD1d+ cells acquire CD34 expression and develop into HE (Fig. 2A), suggesting that either there is significant unappreciated heterogeneity within the CD1d+/CDX4+ mesoderm, or that the development of non-hematopoietic lineages from a common precursor, similar to the hemangioblast (Choi et al., 1998), which possibly serves a supportive role for definitive hematopoietic development.

It also remains to be determined which mesodermal lineage(s) arise from CD1d+ mesoderm. It will be of great interest to determine if there are functional differences in the CD34+ endothelial progeny from CD1d+ and CD1d−/− mesoderm. For example, our scRNAseq analyses show that the CDX4− cluster is enriched in cardiac genes such as TBX2/4 and HAND1/2 (Supplementary Table 2). One possibility is that CD1d−/− mesoderm harbors endocardial potential, which is consistent with observations that early CDX1(1/4) expression is negatively correlated with cardiogenic potential in vivo and in vitro (Lengerke et al., 2011).

Finally, the CDX4hi mesodermal population we identified exhibits enrichment for HOXA1 and HOXA3, of which the latter is a regulator of cardiogenic potential (Sturgeon et al., 2014). Here, we show that the combined markers of KDR, CD235a, and CD1d can identify a hemogenic mesodermal population that harbors exclusively extra-embryonic-like hematopoietic potential (Sturgeon et al., 2017). Human definitive hematopoietic specification from pluripotent stem cells is regulated by mesodermal expression of CDX4. Blood 129 (22), 2988–2992.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102808.
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