Generation and Characterization of a Homogenous Population of Early Mesoderm Cells Using STEMdiff™ Mesoderm Induction Medium

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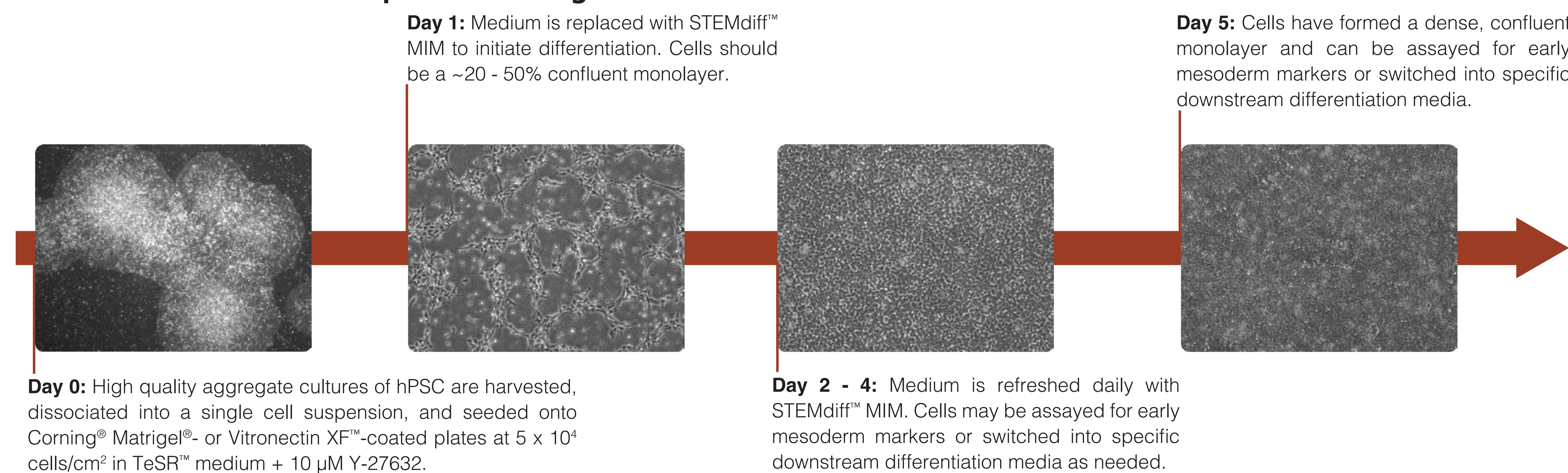
Introduction

Highly efficient protocols to generate mesodermal cell types from human pluripotent stem cells (hPSCs) have been challenging to establish, however, the most successful protocols have generally required a stepwise approach that mimics development. To support the robust induction of the first stage of differentiation into early mesoderm, we have developed STEMdiff™ Mesoderm Induction Medium (MIM), a defined, xeno-free medium that generates a homogeneous population of early mesoderm cells from hPSCs using a fast and simple protocol. Mesoderm induction was efficient and consistent across multiple human embryonic stem (ES) cell and induced pluripotent stem (iPS) cell lines. Early mesoderm cells generated using MIM were capable of further downstream differentiation into endothelial cells (lateral plate mesoderm) and mesenchymal-like cells (paraxial mesoderm).

Methods

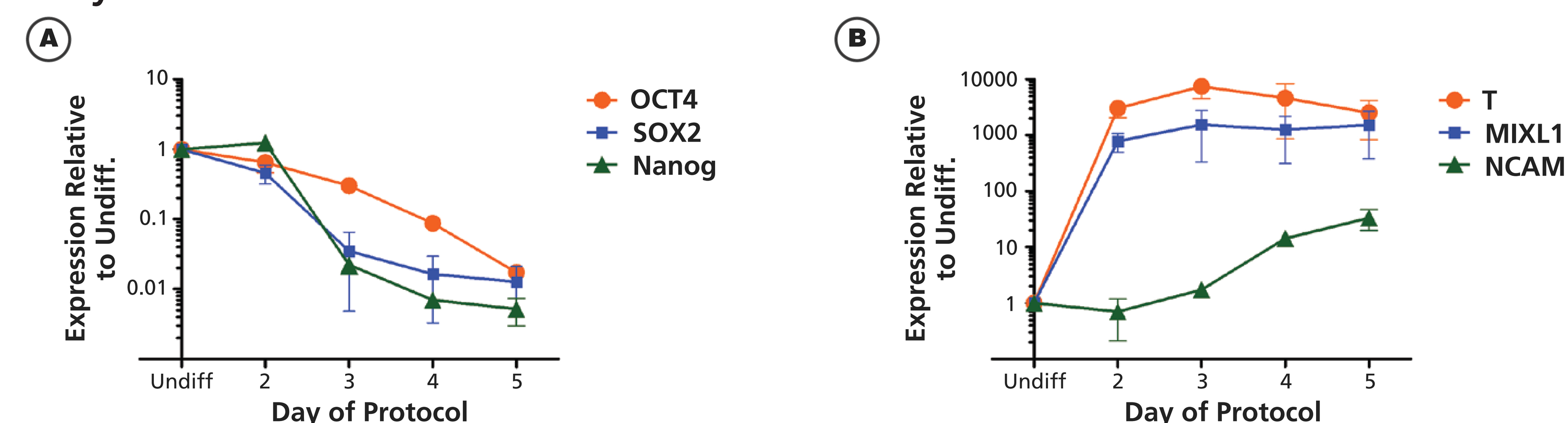
Human ES cell lines (H1, H9) and human iPS cell lines (WLS-4D1, WLS-1C, STiPS-M001, STiPS-F016) were maintained in either mTeSR™1 or TeSR™-E8™ using standard aggregate culture methods and differentiated in MIM using the specific protocol in **Figure 1**. The expression of markers including pan-mesodermal marker Brachyury (T) and the undifferentiated cell marker OCT4 were monitored throughout differentiation using Q-PCR. At day 5 of the protocol, early mesoderm cells were characterized using flow cytometry to assess undifferentiated cell and early and later mesoderm markers. The ability to differentiate into downstream lineages was tested between day 3 and 5 of the protocol as described in **Figures 5 and 6**.

FIGURE 1: Differentiation protocol using STEMdiff™ Mesoderm Induction Medium



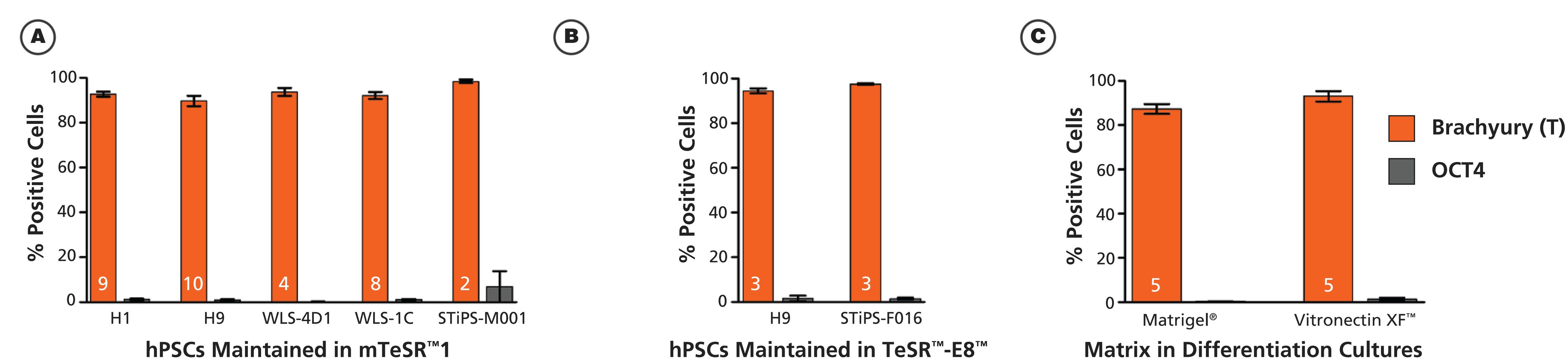
Results

FIGURE 2: Success of differentiation is assessed by the loss of undifferentiated cell markers and the gain of early mesoderm markers



Human PSCs (H9, 4D1) were differentiated using STEMdiff™ MIM and differentiation kinetics were monitored using Q-PCR. Expression for each gene is normalized to the housekeeping gene TBP and then expressed relative to the expression level in undifferentiated hPSCs (mean ± SEM, n = 2). **(A)** Markers of undifferentiated cells start to be down-regulated between day 2 and 3 of the protocol. OCT4 is down-regulated more noticeably between days 3 and 4, and is essentially absent by day 5. **(B)** Markers of early mesoderm (T, MIXL1) are up-regulated rapidly by day 2 and remain highly expressed until day 5. NCAM expression is up-regulated slightly later, at days 4 and 5. The same samples were used to analyze markers in **(A)** and **(B)**.

FIGURE 3: Differentiation to early mesoderm is highly efficient and reproducible in multiple human ES and iPS cell lines

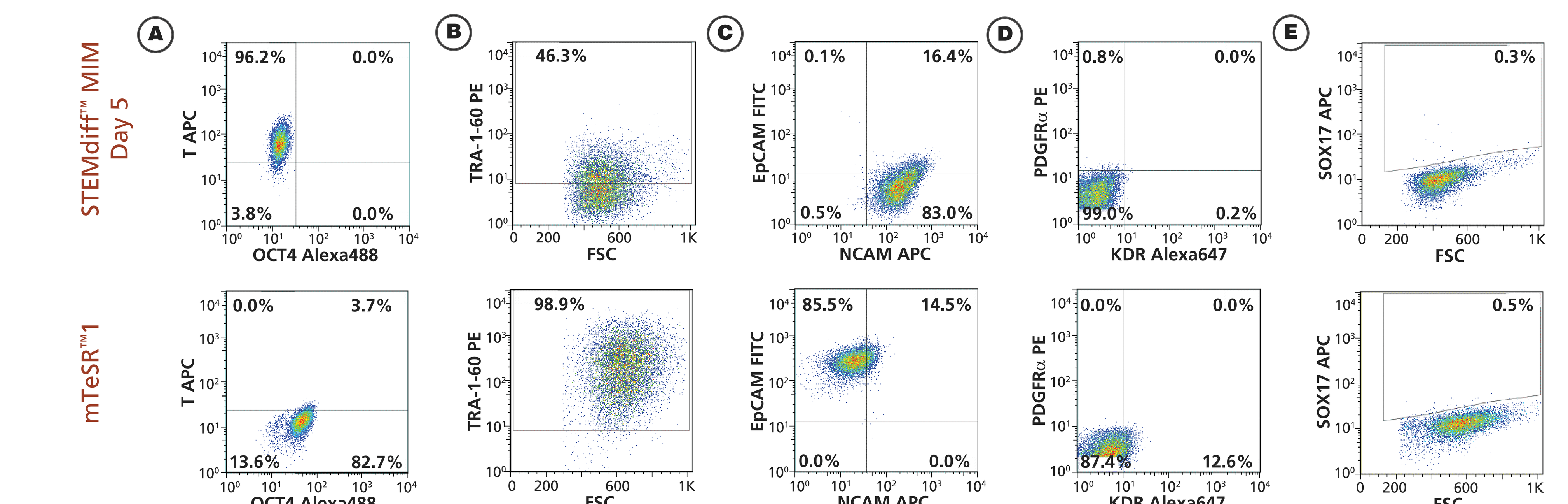


hPSCs were maintained in **(A)** mTeSR™1 or **(B)** TeSR™-E8™ and differentiated using STEMdiff™ MIM. Flow cytometry analysis on day 5 of the protocol shows the percentage of cells positive for T and OCT4 (mean ± SEM). The number of experiments for each cell line is indicated in the bar for T. **(C)** Mesoderm differentiation on Matrigel® or Vitronectin XF™ is comparable.

Summary

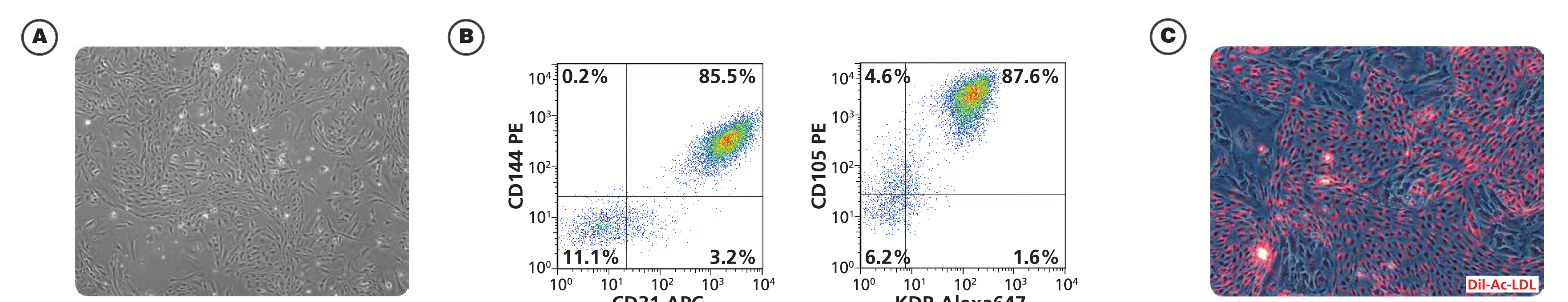
- STEMdiff™ Mesoderm Induction Medium (MIM) promotes differentiation of a homogenous population of early mesoderm cells from hPSCs.
- Differentiated cells generated by the MIM protocol do not express markers of undifferentiated cells, later mesoderm subtypes, or definitive endoderm, indicating early mesoderm induction.
- Fast and simple mesoderm differentiation protocol is compatible with hPSCs maintained in mTeSR™1 or TeSR™-E8™ and can be performed on either Matrigel® or Vitronectin XF™ matrix.
- Early mesoderm cells generated using STEMdiff™ Mesoderm Induction Medium can be used for further differentiation into downstream cell types such as endothelial and mesenchymal cells.

FIGURE 4: Phenotypic characterization of early mesoderm cells generated with STEMdiff™ Mesoderm Induction Medium



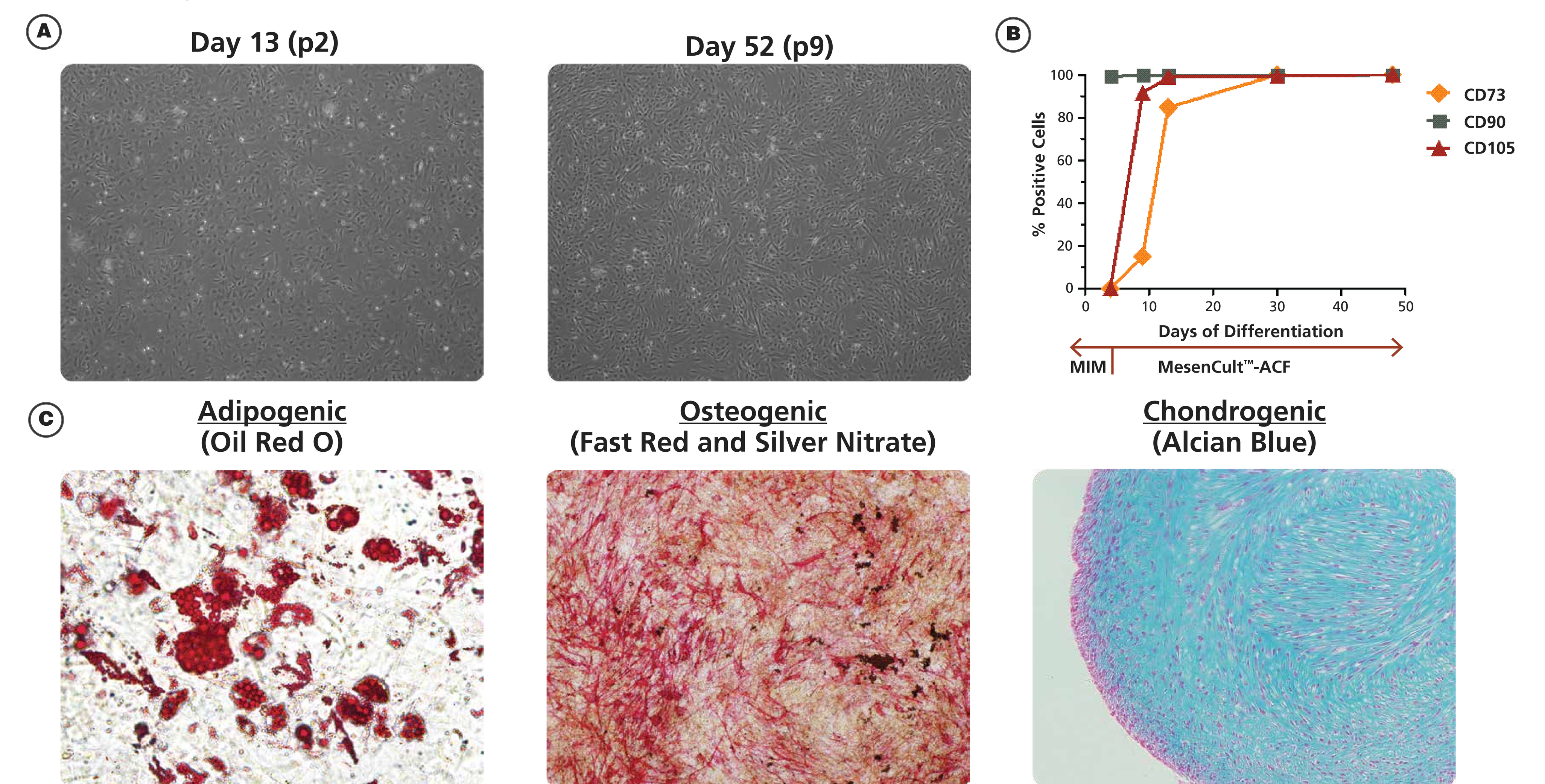
Representative flow cytometry analysis showing marker expression of cells (H9) on Day 5 of the STEMdiff™ MIM protocol (top) and control cells cultured continuously in mTeSR™1 (bottom). Gates are set based on isotype controls. **(A)** After MIM treatment, cells highly express T and have shut off OCT4 expression. Undifferentiated cells highly express OCT4 and do not express T. **(B)** TRA-1-60 is downregulated on early mesoderm differentiated cells compared with undifferentiated cells. **(C)** During early mesoderm differentiation, cells switched cell adhesion molecule expression from EpCAM⁺ to NCAM⁺, indicating epithelial-mesenchymal transition. **(D)** Lateral plate mesoderm marker KDR and paraxial mesoderm marker PDGFRα are not expressed on either cell type. **(E)** Definitive endoderm marker SOX17 is not expressed on either cell type.

FIGURE 5: STEMdiff™ Mesoderm Induction Medium generates early mesoderm cells capable of differentiating to endothelial cells



At day 3 of the MIM protocol, medium was changed to endothelial differentiation medium (Tan *et al.*) which was replaced every second day. After 4 days in endothelial differentiation medium, cells were harvested and enriched using EasySep™ Human CD34 Positive Selection Kit (STEMCELL Technologies) and then replated onto collagen-coated plates with EGM-2 (Lonza) + 50 ng/mL VEGF. Data is shown from a representative experiment using STiPS-F016 cells. **(A)** Endothelial morphology (40X magnification) observed 5 days after replating into EGM-2 medium. **(B)** Flow cytometry analysis for endothelial cell markers showing high expression 5 days after replating cells into EGM-2. Gates are set based on isotype controls for each marker. **Left:** Co-staining of CD31 (also known as PECAM) and CD144 (also known as VE-cadherin); **Right:** Co-staining of KDR (also known as VEGFR2) and CD105 (also known as endoglin). **(C)** Cells that uptake LDL exhibit endothelial-like morphology. Image shows overlay of bright field and Dil-Ac-LDL in red (40X magnification).

FIGURE 6: STEMdiff™ Mesoderm Induction Medium generates early mesoderm cells capable of differentiating to mesenchymal-like cells



At day 5 of the MIM protocol, medium was changed to MesenCult™-ACF (STEMCELL Technologies). Cells were passaged as required using MesenCult™-ACF Dissociation Kit (STEMCELL Technologies) and seeded at 0.5 - 5 x 10⁴ cells/cm² onto matrix-coated plates. Data is shown from a representative experiment using H1 cells. **(A)** Mesenchymal cell-like morphology seen after a total of 13 and 52 days in differentiation media (MIM followed by MesenCult™-ACF). Cells are at p2 and p9 respectively. **(B)** Summary of flow cytometry analysis of mesenchymal cell markers throughout differentiation. Mesenchymal cell markers CD73 and CD105 are expressed during differentiation after cells adopt a mesenchymal-like morphology. CD90 is expressed on undifferentiated cells (not shown) as well as early mesoderm cells and mesenchymal cells, and is therefore not specific for mesodermal differentiation. **(C)** Mesenchymal-like cells generated after MIM treatment differentiate under appropriate differentiation conditions into adipocytes, osteogenic cells, and chondrocytes, as visualized by Oil Red O, Fast Red and Silver Nitrate, and Alcian Blue stains respectively.

Reference: Tan *et al.* Efficient Derivation of Lateral Plate and Paraxial Mesoderm Subtypes from Human Embryonic Stem Cells through GSKi-Mediated Differentiation. Stem Cells and Development 2013: 22(13) 1893-1906.

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