Supplementary Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables.

Supplemental Experimental Procedures

Cell lines

Human H1 ESCs at passage 40-50 were cultured on Matrigel-coated plates in mTeSR1 medium (Stem Cell Technologies).

Coculture of hESCs on AGM-S3 cells to induce hematopoietic differentiation

Undifferentiated hESC clumps (approximately 30 colonies per well of a 6-well plate or 180 clumps in a 10 cm dish), each consisting of 500 to 2,000 cells per clump, were physically picked under a microscope and transferred onto irradiated AGM-S3 cells. Culture medium composed of IMDM containing 10% fetal bovine serum (FBS) (BI) was supplemented with 5.5 μg/mL human transferrin (Sigma), 2 mM L-glutamine, 50 μg/ml ascorbic acid, and 20 ng/ml vascular endothelial growth factor (VEGF) (Perptech). Culture medium was replaced every day. At day 14, the cocultures were treated with 0.25% trypsin/EDTA solution (GIBCO). Subsequently, whole cells (including multipotent hematopoietic progenitor cells) were collected for future use.

Expansion of $CD34^+CD45^+$ hematopoietic progenitor cells

Similar to our previous studies (Mao B et al, 2016), total cocultured cells were harvested by treatment with 0.25% trypsin/EDTA solution and sorted $CD34^+CD45^+$ cells, and then cultured in a non-adhesion 24-well plate (Costar, plate surfaces resistant to cell adhesion) in IMDM medium with 10% FBS and a cocktail of cytokines favoring hematopoietic progenitor cell development (SCF, 100ng/ml; IL-3, 10ng/ml; IL-6,100ng/ml; Flt3; 5ng/ml; and TPO, 5ng/ml) for 7 days. This method ensured the robust growth of hematopoietic progenitor cells with no growth of stromal cells. After 7 days in culture, a large quantity of hematopoietic progenitor cells was obtained, most of which were of the myeloid phenotype.

Induction of MCs in serum-free culture medium

In the next stage, the hematopoietic progenitor cells were shifted to serum-free MC culture in IMDM and 10% SFEM II (Stem Cells Tech) with the addition of SCF (100ng/ml), Flt-3 (2ng/ml), IL-6 (50ng/ml), and TPO (2ng/ml). Half of the volume of culture medium was replaced with fresh medium twice per week.

MCs Derived from Human Cord Blood $CD34^+$ HSPCs

To generate mucosal-type MCs, we isolated hCB $CD34^+$ HSPCs using $CD34^+$ Dynabeads (Stem Cell Tech; purity >90%) and cultured them in serum-free medium with SCF (100ng/ml) and IL-6 (80ng/ml). Half the volume of culture medium was replaced with fresh medium twice per week.

MCs derived from $CD34^+$c-kit^+ cell populations

Cocultured day8 cells were dissociated with 0.01% trypsin-EDTA solution (Invitrogen) and filtered through a 40 μm nylon mesh to obtain a single-cell suspension. $CD34^+$c-kit^+ cell population was sorted using a MoFlo Astrios High Speed Cell Sorter (Beckman Coulter), and then cultured in a non-adhesion 24-well plate by SFEM II (stem cell) medium and a cocktail of cytokines favoring MC development (SCF,100ng/ml; IL-6, 100ng/ml; and Flt-3, 20ng/ml). Half of the volume of culture medium was replaced with fresh medium twice per week.

Flow cytometry and cell sorting

Cocultured cells were detached with 0.25% trypsin-EDTA solution (Invitrogen) and filtered through a 70 μm nylon mesh to generate a single-cell suspension. Flow cytometry was performed using a FACSCanto II system (BD Biosciences), and data were analyzed using FlowJo software (v10.0.8.). Cell sorting was performed using a MoFlo Astrios High Speed Cell Sorter (Beckman Coulter). The antibodies used are presented in Table S2.

Low level RNA-seq data processing

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. A PureLink RNA Micro kit (Invitrogen) was used to extract total RNA from sorted cells. cDNA was synthesized using an iScript cDNA Synthesis kit (Bio-Rad). All real-time PCR assays were performed using FastStart Universal SYBR Green Master (Roche) and an iQ5 thermal cycler (Bio-Rad). Expression of target genes was normalized against that of *GAPDH*. All reactions were performed in triplicate. The primers used are listed in Table S3.

Morphological observations and immunofluorescence staining of MCs

Mature MC culture cells were centrifuged and mounted onto glass slides. Cytospin preparations were then stained with MGG, acidic toluidine blue, and Alcian blue. For MC-specific tryptase and chymase assays, an immunofluorescence staining method was used, as previously reported (Ma F et al, 2008). The antibodies used are listed in Table S4.

Confirmation of MCs

MCs were confirmed to be 90% pure by immunofluorescence staining for c-kit and tryptase. Cells were used for further studies when the morphology was 100% uniform.

Activation of MCs

For IgE stimulation, MCs at 12 weeks of MC culture were cultured for 3 days in medium supplemented with 4 µg/ml IgE. To assess activation, cells were washed, suspended in Thyrode's Solution, and pre-incubated for 5 min at 37ºC. For stimulation, 10 µl CRA-1 or control mouse IgG, substance P (MCE), or compound 48/80 (Sigma)were added to a 90 µl cell suspension (4×10^4 cells/ml) in 96-well plates and incubated for a further 30 min at 37ºC. The reaction was stopped by adding 100 µl ice-cold buffer. Cells were separated by centrifugation at 300 x g for 7 min at 4℃, and the supernatant was collected. The cell pellet was resuspended in 200 µl buffer containing 0.5% Triton-X and 0.1% bovine serum albumin (BSA), snap frozen in liquid nitrogen, and thawed four times. After centrifugation at 12,000 x g for 15 min at 4ºC, soluble extract was collected. Histamine levels were measured using a histamine ELISA kit (Abcam).

Intracellular histamine assay

Intracellular histamine concentration was measured by quantifying histamine levels in cell lysates using the same ELISA histamine assay kit. Briefly, 500 MCs at 12 weeks of MC culture were washed and pooled in 0.5 ml PBS. The cell suspensions were then snap frozen in liquid nitrogen and quickly returned to a 37ºC water bath to thaw. The freeze-thaw cycle was repeated twice, and cell lysate histamine content was then measured according to the manufacturer's protocol.

Cytokine production by stimulated MCs

MCs (100,000/mL) at 12 weeks were stimulated with IgE followed by anti-IgE-R for 24 h before the supernatant was collected into a micro-centrifuge tube and frozen at -80°C for cytokine quantification.

Multiplex cytokine analysis

Cytokine concentrations in the culture supernatants were measured using Meso Scale human assays for the indicated cytokines per the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). Seventeen cytokines were assessed using a human cytokine kit (U-PLEX Biomarker Group 1 Assay containing IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL23p40, IL-13, IL-17α, TNF-α, IFN-Y, G-CSF, GM-CSF, MCP-1, and MIP-1β) on a SECTOR Imager instrument (Meso Scale Discovery, Rockville, MD, USA) according to the manufacturers' protocols.

Cell preparation

Fresh cells were harvested and resuspended at 1×10^5 cells/ml in 1×PBS and 0.04% BSA. Cell concentration was determined using a Countess® II Automated Cell Counter, and the volume was adjusted to obtain the target cell concentration before proceeding with the 10x Genomics® Single Cell Protocol.

Batch corrections among samples

Considering the batch effects among samples in day 5 and day 5_5, FindIntegrationAnchors and IntegrateData contained in the Seurat package were used to align datasets across different batches and merge samples into one object with default parameters.

Identification of differentially expressed genes (DEGs)

The FindAllMarkers function of Seurat was used to identify DEGs among different clusters. The parameter “only.pos” was set as “TRUE”, which returned the upregulation gene clusters. A wilcoxon test was used to evaluate the statistical significance of each gene.

Inventory of supplemental information

Table S1. Summary of the sequencing metrics for the two samples.

Table S2. Antibodies used for flow cytometric analysis.

Table S3. Primers used for qRT-PCR analysis.

Table S4. Antibodies used for immunostaining.

Figure S1, Related to Figure 1.

Figure S2, Related to Figure 2.

Figure S3, Related to Figure 3.

Figure S4, Related to Figure 4.

Table S1: Summary of sequencing metrics for the two samples.

Sample	D5	D5_5
Estimated Number of Cells	2167	6734
Mean Reads per Cell	438699	138923
Median Genes per Cell	6206	4209
Number of Reads	950,662,793	935,511,175
Valid Barcodes	0.97	0.97
Q30 Bases in Barcode	0.96	0.96
Q30 Bases in RNA Read	0.90	0.90
Q30 Bases in UMI	0.96	0.96
Reads Mapped to Genome	0.91	0.89
Reads Mapped Confidently to Genome	0.89	0.86
Reads Mapped Confidently to Intergenic Regions	0.06	0.07
Reads Mapped Confidently to Intronic Regions	0.13	0.15
Reads Mapped Confidently to Exonic Regions	0.70	0.65
Reads Mapped Confidently to Transcriptome	0.66	0.61
Reads Mapped Antisense to Gene	0.009	0.01
Fraction Reads in Cells	0.82	0.83
Total Genes Detected	17116	17288
Median UMI Counts per Cell	48199	23711

Table S2: Antibodies used for Flow cytometry

Name	Format	Brand	Cat. No.	Isotype	Clone
c-kit	PE	eBioscience	12-1179-42	Ms IgG1, κ	YB5.B8
c-kit	APC	eBioscience	17-1179-42	Ms IgG1, κ	YB5.B8
CD34	APC	BD	555824	Ms IgG1, κ	581
CD34	PE-Cy7	BD	560710	Ms IgG1, κ	581
CD43	PE	BD	560199	Ms IgG1, κ	1G10
CD45	PE	BD	555483	Ms IgG1, κ	HI30
CD45	PE-Cy7	BD	557748	Ms IgG1, κ	HI30
CD309/Flk1	PE	BioLegend	359904	Ms IgG1, κ	7D4-6
CD13	PE	BD	561599	Ms IgG1, κ	WM15
CD203c	PE	MACS	130-099-238	Ms IgG1, κ	FR3-16A11
CD88	PE	BD	SC-53796	Ms IgG1, κ	W17/1
FcεRIα	PE	eBioscience	12-5899-42	Ms IgG2b, κ	AER-37
CD81	PE	BD	551112	Ms IgG1, κ	JS-81
HLA-DR	APC	BD	559866	Ms IgG2a, κ	G46-6
CD31	PE	BioLegend	303106	Ms IgG1, κ	WM59
CD144	Alexa Fluor® 647	BD	561567	Ms IgG1, κ	55-7H1
FVS520		BD	564407	Ms IgG1, κ	
7-AAD		BD	559925	Ms IgG1, κ	

Table S3: Primers used for qRT-PCR

Gene	Direction	Sequences
c-kit	Forward	CACCGAAGGAGGCACTTACACA
	Reverse	TGCCATTCACGAGCCTGTCGTA
Tryptase	Forward	GTGACGCAAAATACCACCTTGGC
	Reverse	CCATTCACCTTGCACACCAGGG
Chymase	Forward	TGTGGGCAATCCCAGGAAGACA
	Reverse	GACCGTCCATAGGATACGATGC
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
	Reverse	ACCACCCTGTTGCTGTAGCCAA

Table S4: Antibodies used for Immunostaining

Name	Manufacturer	Species	Cat. no.
c-kit(K963)	IBL	Rabbit to human	18101
Tryptase(E-17)	Santa Cruz	Goat to human	SC-17039
Chymase(CC1)	Abcam	Mouse to human	ab2377
FITC-conjugated secondary Ab	Jackson Immuno Research	Donkey to Goat	705-095-003
Cy3-conjugated secondary Ab	Jackson Immuno Research	Donkey to Mouse	715-165-150
Cy3-conjugated secondary Ab	Jackson Immuno Research	Donkey to Rabbit	711-165-152

Figure S1

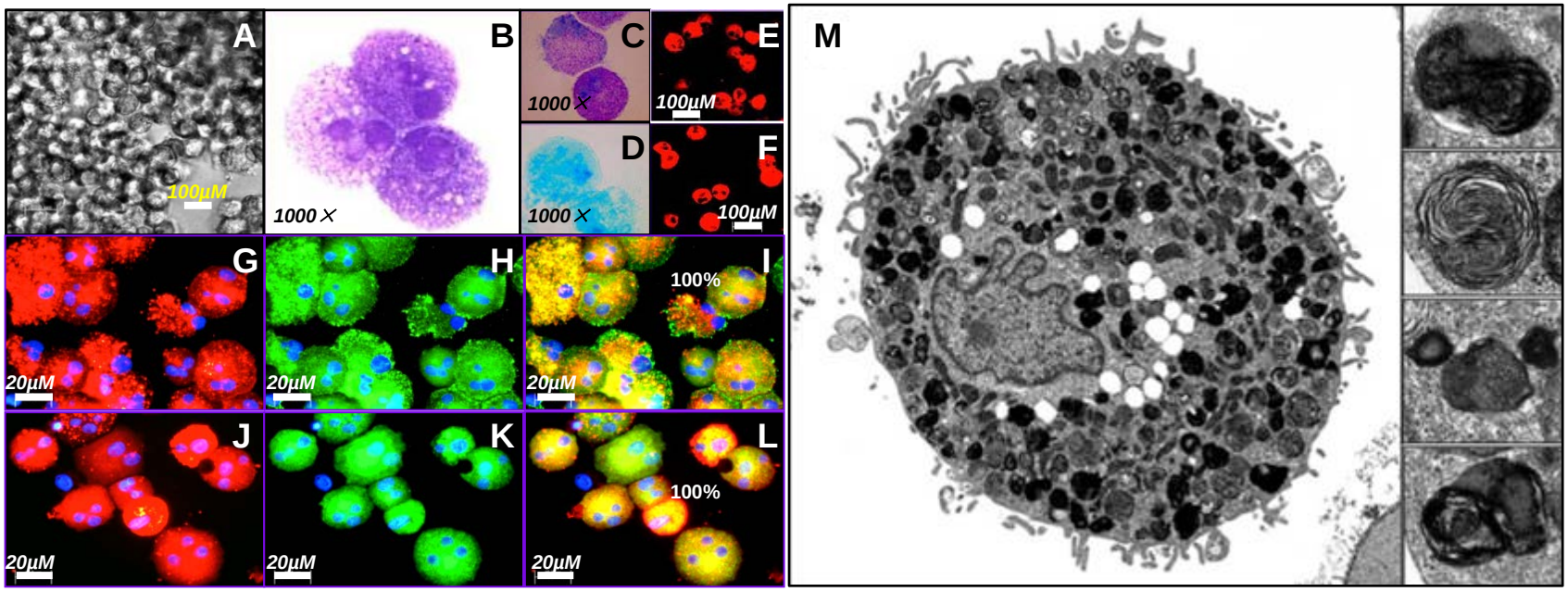

Figure S1: Phenotype of MCs derived from hESC/AGM-S3 coculture system. (A) hESC-MC growth under a microscope. Staining of hESC-MCs (20 weeks in SF-MC culture) with (B) May-Grunwald Giemsa, (C) Toluidine blue, (D) Alcian blue, (E) Carboxypeptase A, and (F) Cathepsin G. (G-L) hESC-MC maturity after 10 weeks in SF-MC culture was further confirmed by the 100% co-expression of (G) c-kit, (H) Tryptase, (I) Merged, (J) Chymase, (K) Tryptase, and (L) Merged. (M) Transmission electron microscope images of hESC-MCs after 20 weeks in SF-MC culture. The four small insets at the right side show various types of MC-specific granules.

Figure S2

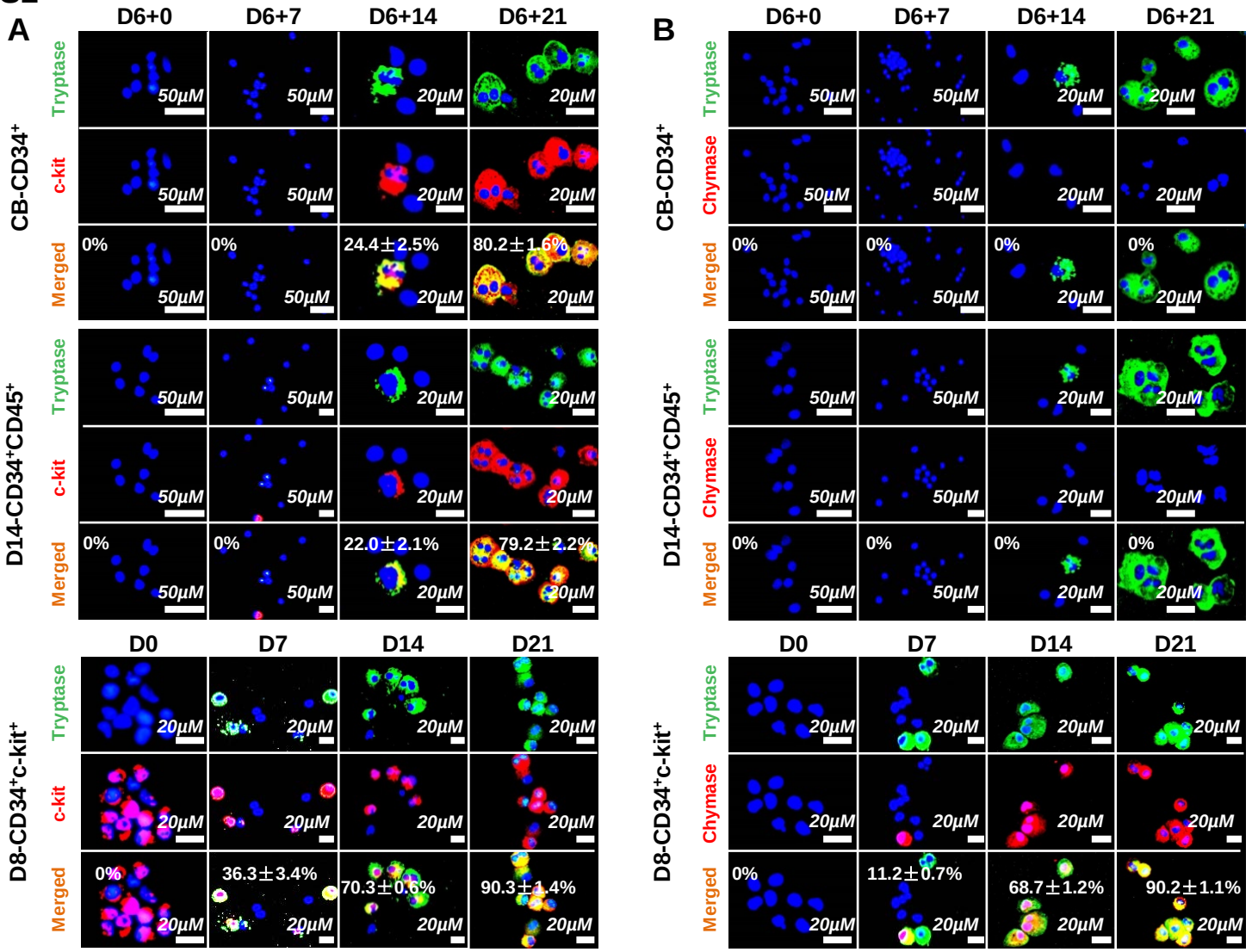

Figure S2: Tryptase⁺/Chymase⁺ MCs only appeared in daughter cells of coculture day8 CD34⁺c-kit⁺ cell populations. A high purity (~90%) of CD34⁺c-kit⁺ population derived c-kit⁺/tryptase⁺/chymase⁺ MCs could be observed after 3 weeks in SF-MC culture. Photos represent immunostaining for (A) c-kit and tryptase double-positive MCs, and (B) tryptase and chymase double-positive MCs, over the time course of cultivation derived from the three different MCs.

Figure S3

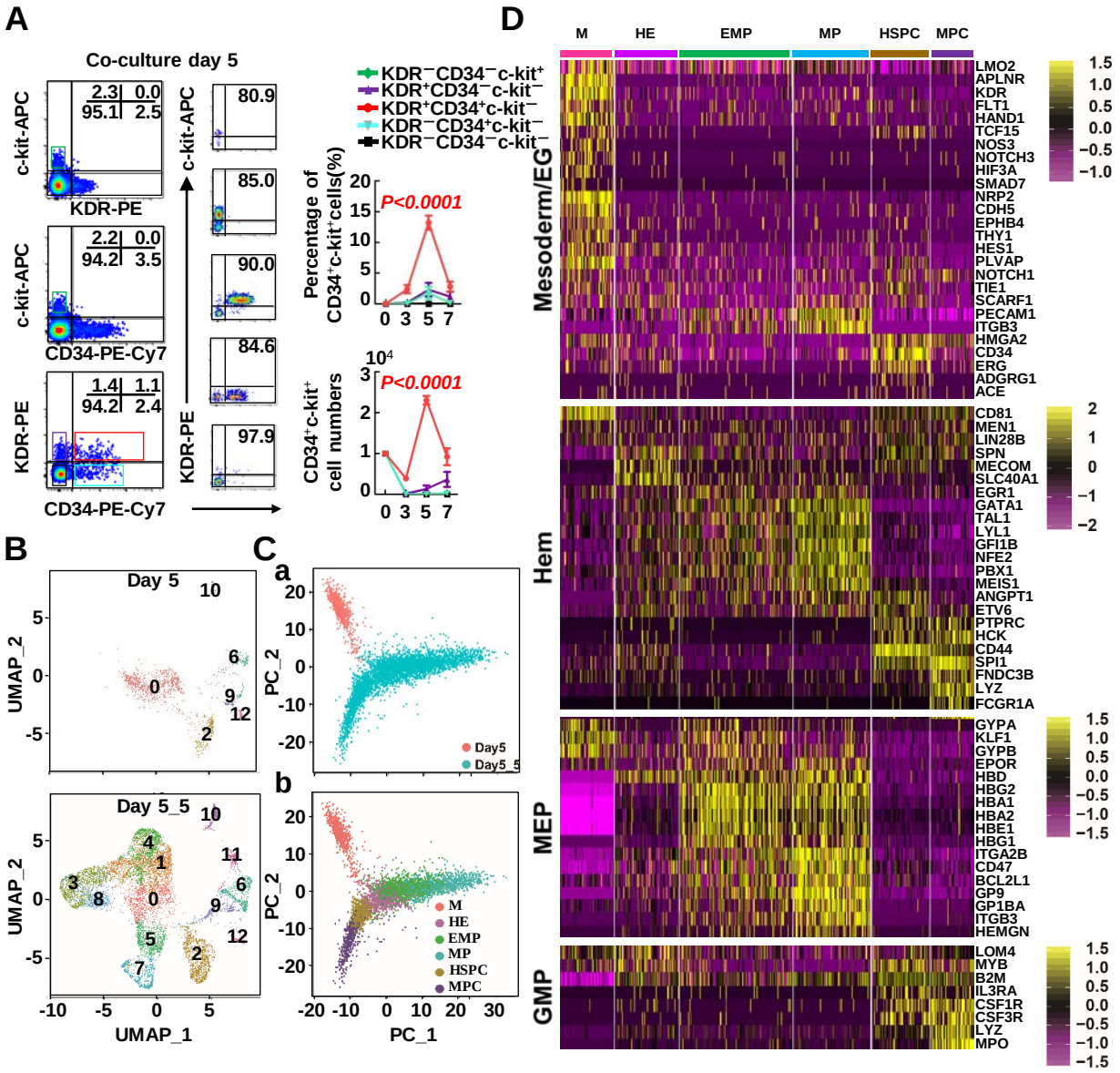

Figure S3: CD34⁺c-kit⁺cell population primarily derived from the KDR⁺CD34⁺ cell fraction and shared multi-directional development potential. (A) Flow cytometric analysis of KDR⁻CD34⁻c-kit⁻, KDR⁺CD34⁻c-kit⁻, KDR⁻CD34⁺c-kit⁻, KDR⁺CD34⁺c-kit⁻, and KDR⁻CD34⁻c-kit⁺ cells sorted from hESC/AGM-S3 co-cultures on day 5. Percentages and absolute numbers of CD34⁺c-kit⁺ cells derived from each cell fraction are shown. Error bars represent mean ± SD of samples from at least three independent experiments. (B) Identification of cell populations in (a) D5 and (b) D5_5 visualized by UMAP. Each dot represents one cell, and colors represent the cell clusters as indicated. (C) PCA plot of (a) D5 and D5_5 samples and (b) PCA plot of Mesoderm, HE, EMP, MP, HSPC, and MPC clusters. (D) Heatmap showing scaled expression of mesoderm, EG, Hem, MEP, and GMP-related genes in the six clusters.

Figure S4

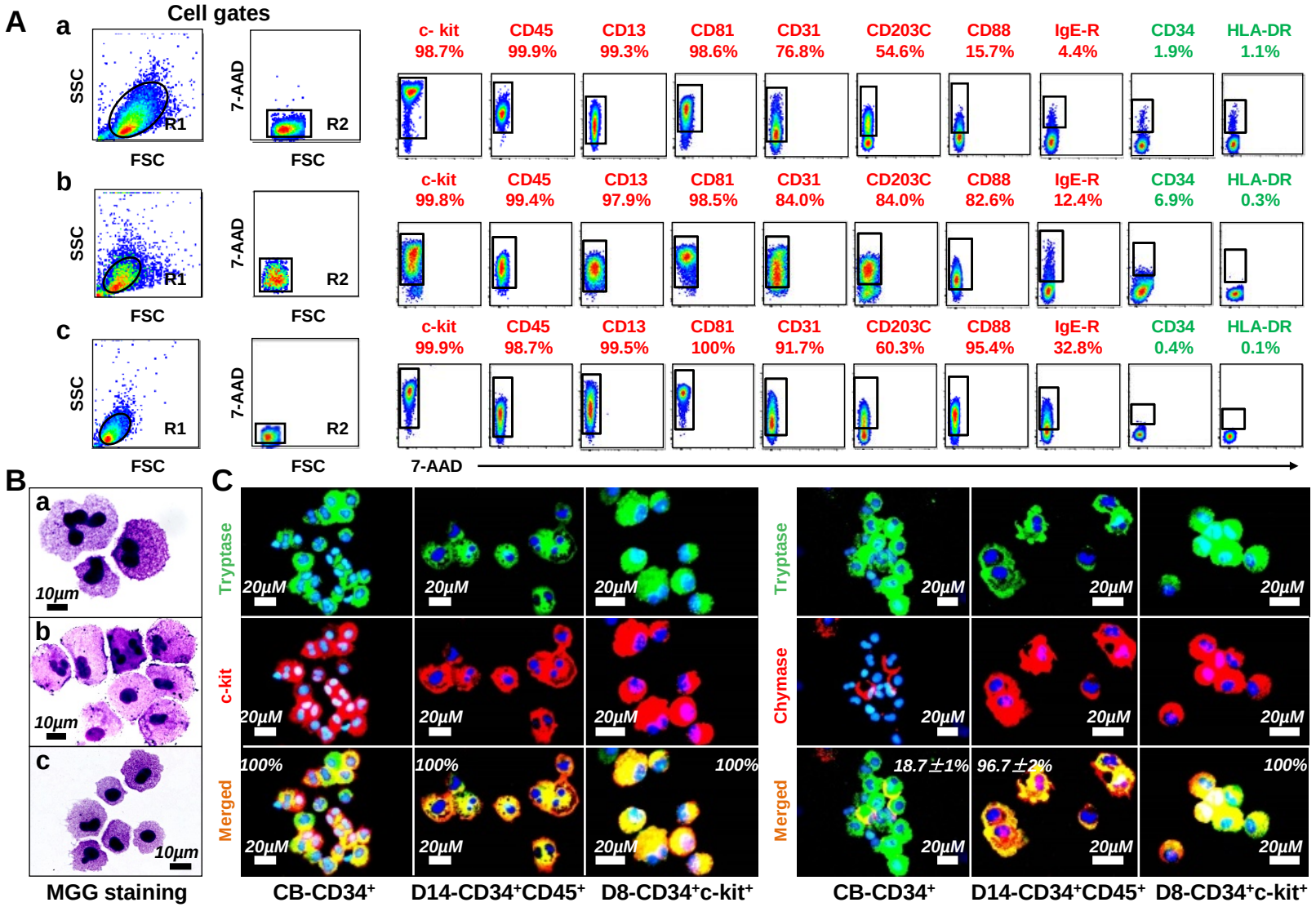

Figure S4: Morphological characters and molecular profiling of surface marker expression among the three different MCs. (A) Representative dot plots of surface marker expression of different protocol derived mature MCs, including (a) CB CD34⁺, (b) coculture day14 CD34⁺CD45⁺, (c) and coculture day8 CD34⁺c-kit⁺ cells (12 weeks in SF-MC culture). (B) MGG analysis showing typical morphology of MCs from (a) CB-CD34⁺, (b) coculture day14 CD34⁺CD45⁺, and (c) coculture day8 CD34⁺c-kit⁺. Independent experiments, n = 3; mean ± SD. Scale bars, 10 μm. (C) Immunostaining analysis showing expression of human c-kit, Tryptase, and Chymase in MCs derived from different pathways. Independent experiments, n = 3; mean ± SD. Scale bars, 20 μm.