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BRIEF REPORT

Hematopoietic Stem/Progenitor Cells, Generation of Induced Pluripotent Stem Cells, and Isolation of Endothelial Progenitors from 21-23.5 Year Cryopreserved Cord Blood.

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Running Title: Long-Term Cryopreservation of Cord Blood

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Abstract

Cryopreservation of hematopoietic stem (HSC) and progenitor (HPC) cells is crucial for cord blood (CB) banking and transplantation. We evaluated recovery of functional HPC cryopreserved as mononuclear or unseparated cells for up to 23.5 years compared to pre-freeze values of the same CB units. Highly efficient recovery (80-100%) was apparent for granulocyte-macrophage and multipotential hematopoietic progenitors, although some collections had reproducible low recovery. Proliferative potential, response to multiple cytokines, and replating of HPC colonies was extensive. $CD34^+$ cells isolated from CB cryopreserved for up to 21 years had long-term (≥ 6 month) engrafting capability in primary and secondary immunodeficient mice reflecting recovery of long-term repopulating, self-renewing HSC. We recovered functionally responsive $CD4^+$ and $CD8^+$ T-lymphocytes, generated induced pluripotent stem (iPS) cells with differentiation representing all 3 germ cell lineages in vitro and in vivo, and detected high proliferative endothelial colony forming cells, results of relevance to CB biology and banking.

Introduction

The first cord blood (CB) transplant saved the life of a young patient with Fanconi anemia using HLA-matched sibling CB cells,[1] a procedure possible by identification and cryopreservation of transplantable hematopoietic progenitor (HPC) and stem (HSC) cells in CB.[2] Over 20,000 CB transplants have treated the same malignant and non-malignant disorders as bone marrow (BM).[3-8] CB transplantation is possible due to CB banks, and how long CB can be stored in a cryopreserved state with efficient recovery of HSC and HPC is critical for CB banking. We reported highly efficient recovery of CB HPC after 5,[9] 10,[10] and 15[11] years, and recovery of HSC after 15 years.[11] We now report efficient recovery of functional HPC up to 21-23.5 years, with more in depth studies on CB HSC engraftment in immune deficient mice, recovery of responsive T cells, generation of induced pluripotent stem (iPS) cells,[12-14] and detection of endothelial colony forming cells (ECFC).[15]

Materials and Methods

CB cells were scheduled for discard.[2] The study was approved by Institutional Review Board of Indiana University (IU). Cryopreservation, thawing and plating were as reported.[2,9-11] CB was assessed within 36 hrs of collection. Cells were either separated into a mononuclear (MNC) fraction (Ficoll-Hypaque, Pharmacia) and aliquoted into cryotubes (Nalge Nunc) or left unseparated and aliquoted into cryo-freezer bags,[2,16,17] in 10% Dimethylsulfoxide and 10% autologous plasma for eventual analysis of HPC recovery. Percent recovery from MNC or unseparated cryopreserved cells was based on total pre-freeze cells per volume of the exact same CB unit.[2,9-11] After thaw of unseparated cells, $CD34^+$ cells were magnetic bead separated,[11] for HSC engraftment and iPS cell generation studies. $CD4^+$ and $CD8^+$ T-lymphocytes were separated from the $CD34^+$-depleted cells and stimulated on plates pre-coated with anti-CD3 (OKT3, 0.5µg/ml) and anti-CD28 (clone CD28.2, 1µg/ml) with 10% FBS, 50µM 2ME and 10ng/ml IL-15 as described.[18]

Immune deficient mouse assay for human CB donor chimerism was as reported,[11] except that recipients were NOD/SCID/IL-$2Rg^{null}$ (NSG).[19]

iPS cell generation. At IU, $CD34^+$ cells isolated from thawed unseparated cells were grown with 10% FBS, 10ng/ml human (h) SCF, 10ng h Flt3-ligand, and 10ng h Thrombopoietin/ml for 3 days. At day 4, cells were spin-infected (2200 rpm; 45 min.) with concentrated lenti-viral vectors Sox2-Oct4-EGFP and cMyc-Klaf4 (pc DNA-HIV-CS-CGW, provided by Dr. P. Zoltick, Children's Hospital, Philadelphia) (Supplemental Figure 1) in α-MEM medium with polybrene

(Sigma-Aldrich). Medium was replaced at 6 days with above cytokines. At day 7, cells were transferred to mitotically-inactivated MEFs and cultured as for hESCs.[20] iPS cells were also generated at Johns Hopkins using retroviruses expressing Oct4, Sox2, Klf4 and c-Myc.[12]

ECFC assay was performed with MNCs isolated from thawed unseparated CB.[15]

Results and Discussion

HPC recovery. We show efficient recovery of HPC from 23 different collections of MNCs thawed from vials after 21-23.5 years (Figure 1A) compared to the exact same unit's pre-cryopreservation numbers, a recovery similar to 10 and 15 year thaws, that assessed the same plus additional CB units. A range of recoveries was evident, but values were similar whether the same samples were assessed 3 times over 3 years, two times over 1-3 years, or twice on the same day (data not shown). Recovery of CFU-GM and CFU-GEMM from unseparated cryopreserved cells (N=3) was greater than 80% (Data not shown), and consistent with recovery from MNCs. It is not clear why some samples resulted in low efficiency recovery, but assessing the recovery of stored cells by thawing a small sample prior to their possible use in a clinical transplant setting could help identify low recovery CB units, and a decision made as to whether or not to use that unit. Proliferation of HPC was high (Figure 1B) and within range for fresh CB.[2, 9-12] Thawed CB is highly responsive to increased colonies from immature HPC when GM-CSF plus SCF and/or FL are used to stimulate them, compared to that of only GM-CSF (Figure 1C) demonstrating retention of immature HPC.[9] Thawed CB contain HPC colonies that can be replated (Figure 1D), suggesting maintenance of HPC with limited self-renewal capacity.[21] Secondary CFU-GM/M colonies formed from single replated CFU-GM/M colonies. CFU-GEMM colonies gave rise to secondary colonies of CFU-GEMM, erythroid progenitors, CFU-GM, and CFU-M.

HSC activity. Using different CB collections cryopreserved as unseparated cells, isolated $CD34^+$ cells efficiently engrafted NSG mice for 6-7 months (Figure 1E). In 2 experiments, BM cells from engrafted chimeric mice repopulated secondary mice for 6 months. While we

demonstrated engrafting capability of thawed CB after 15 years of storage using first generation NOD/SCID mice,[11] those mice did not allow long-term primary engraftment or secondary repopulation. Thus, the current study greatly extends previous findings, and demonstrates recovery of long-term repopulating and self-renewing HSC. We could not calculate percent recovery of HSC as this assay was not available when cells were cryopreserved, but this engraftment is similar to fresh CB HSC.[11]

T cell activity. Attaining vigorous T cell responses against common viral pathogens is critical for survival after CB transplantation.[22] CB T cells are almost exclusively naive cells, with few effector or memory cells.[18] CB T cells are 'immature' compared to adult T cells due to impaired cytokine production and diminished lytic activity.[22,23] To verify immune capability, $CD4^+$ and $CD8^+$ T-lymphocytes, purified from unseparated CB stored up to 21 years, were activated as assessed by CD3/CD28-induced expression of CD25, (Figure 1F). This demonstrated recovery of functional T cell subsets.

iPS cells. iPS cells are generated from different cell sources,[24,25] including fresh CB,[12-14] and CB cryopreserved for 5-8 years.[12,14] We generated iPS cells from CB cryopreserved for up to 21 years using Oct4, KLF-4, Sox2, and c-Myc reprogramming with lentiviral vector transduction of $CD34^+$ cells at IU (Figure 2A). iPS cell colonies stained positive for OCT4, NANOG, TRA-1-60, SSEA4 and alkaline phosphatase (Figure 2B). Quantitative RT-PCR demonstrated reprogramming via expression of endogenous *OCT4*, *SOX2*, and *NANOG* in comparison to H9 ESC cell line and $CD34^+$ cells from which iPS cells were generated (Figure 2C). Unmethylated OCT4 promoter in two iPS cell lines generated from thawed CB in comparison to enhanced

methylation for $CD34^+$ cells from which iPS cell colonies were derived (Figure 2D), demonstrates early stages of produced cells. Embryoid bodies developed from iPS cells after removal from MEFs (Figure 2E), and expressed ectodermal, mesodermal, and endodermal proteins (Figure 2E). Moreover, injection of iPS cell colonies into testis capsules of immune deficient mice, demonstrated teratomas with ectoderm, mesoderm, and endoderm, confirming reprogramming. Generation of iPS cells[12] at Johns Hopkins with 21 year frozen CB from a different collection, produced cells expressing TRA-1-60, SSEA4, NANOG, and OCT4 (Data not shown), and produced teratomas[12] with expression of endoderm, mesoderm, and ectoderm markers (Figure 2G). These CB-derived iPS cells were differentiated in vitro (Figure 2H). Efficiency of iPS cell generation from thawed CB ranged from 0.027-0.05% per $CD34^+$ cell, similar to cultured CD34+ cells from freshly isolated or shorter term frozen CB.[12] This reprogramming efficiency appears higher than from human adult blood or fibroblastic cells.[12, 14] If iPS cells are found to be of clinical utility, which is not yet clear,[24,25] HLA-typed CB stored in banks could serve as a source of such typed cells.

ECFC. High proliferative ECFC have been identified in CB.[15] MNC from thawed unseparated CB stored frozen for up to 21 years formed ECFC colonies, but their size was smaller than colonies from fresh CB (Figure 2I). ECFC colony numbers from thawed CB (2-$5/10^7$ mononuclear cells) was 1/5 to 1/10 numbers from fresh CB, even when colonies from fresh cells vs. those frozen and stored for up to 3-6 months were assayed. Thus, the freezing procedure that works well for efficient recovery of HPC, may not be optimal for storage of ECFCs. However, ECFCs which can be cryopreserved and recovered may be of value for regenerative medicine, if clinical applicability is proven.[24]

Thus, recovery of HSC, HPC and other early cell types bode well for CB banking and use.

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Author Contributions

HEB, M-RL, NP, ZY, SW, KC, CL, and MCY designed experiments.

HEB, M-RL, GH, SC, NP, Y-JK, CM, ZY, SW, KC, CL, MCY performed research and analyzed and interpreted data.

HEB wrote paper, and M-RL, NP, Y-JK, ZY, SW, KC, CL and MCY and HEB edited the paper.

Studies were supported by grants to HEB, LC, and MCY.

Disclosure of Conflict of Interest

HEB is on the MSAB of Corduse, a cord blood banking company. MCY is a co-founder and consultant to EndGenitor Technologies, Inc.

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Figure Legends

Figure 1. Recovery of nucleated cellularity, HPC, HSC, and immune cells after cryopreservation and long-term frozen storage of CB. (A) Comparative percent recovery of nucleated cells, CFU-GM, and CFU-GEMM compared to pre-freeze numbers for 10, 15, and 21/23.5 years of the exact sample frozen. n = number of different samples thawed for analysis. Results shown as mean ± 1SEM with range of recoveries shown in parentheses. (B) Representative examples of colonies grown from CB thawed after 21-23.5 years in frozen state: (i) CFU-GEMM colony; (ii) CFU-GEMM (left) and CFU-GM (right) colonies; (iii) CFU-GM colony; (iv) CFU-GEMM colony. A Nikon TMS microscope (Nikon Instruments Inc., Melville, NY) was used with PLAN objective 4x and projection lens magnification of 2.5x for a total magnification of 10x at 25°C. We used 35mm film (ASA200), and pictures made with a Nikon HFX-DX automatic 35mm camera system (Nikon Instruments). (C) Ratio of CFU-GM colonies formed after stimulation of CB cells with GM-CSF plus either SCF, FL, or SCF plus FL, divided by number of colonies formed by stimulation of same cells with only GM-CSF. n = number of different CB samples analyzed from cells frozen from 21 to 23.5 years before thaw and analysis. (D) Replating capacity of single CFU-GEMM- or CFU-GM plus CFU-M (macrophage) -colonies recovered from thawed CB cells stored frozen for up to 21 years. Results shown are from a total of over 1000 separately replated colonies each, and designate percent of secondary plates with at least one colony (% replates = top) and range of colonies in secondary dish per single replated colony (= bottom). (E) Analysis of engrafting capacity in sublethally-irradiated NOD-SCID IL-2 receptor gamma chain null (NSG) mice of cells (≥ 90% $CD34^+$) purified from unseparated CB stored frozen for 18-21 years prior to thawing. Each experiment shows chimerism data from a different frozen CB unit. Analysis of human $CD45^+$ chimerism in peripheral blood (PB) or bone

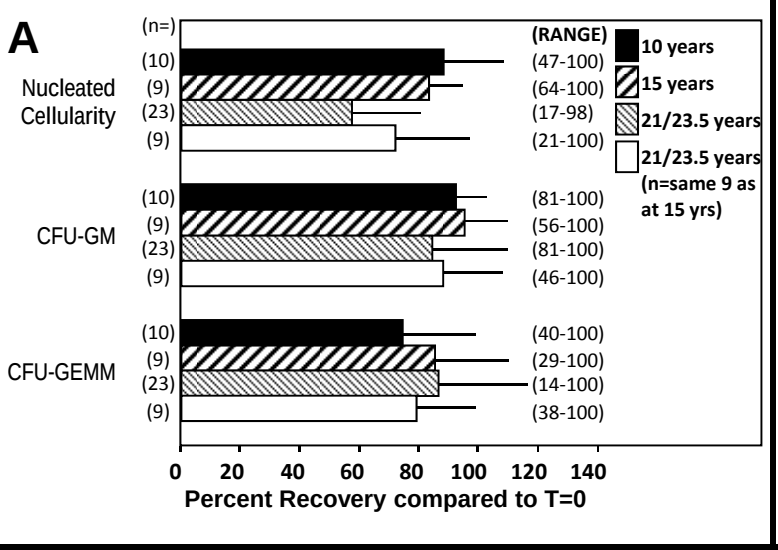
marrow (CB) of primary (1°) mouse recipients with N=number of mice per group, or $CD45^+$ cell chimerism in secondary (2°) recipients of the same mouse strain given the same sublethal irradiation dose. For 2° recipients, each bar represents the number of secondary recipients per pooled BM of 1° recipient mice (For experiment 1 and 2 only). Results are given as mean ±1SEM. (F) Response of $CD4^+$ and $CD8^+$ T lymphocytes, (≥ 98% Pure) isolated from thawed unseparated CB cells stored frozen for 21 years, as assessed by Flow Cytometry, to anti-CD3/CD28 stimulation, as determined by induced expression of CD25, a T cell activation antigen. Shown are experiments from one of two experiments in which different frozen CB units were assessed.

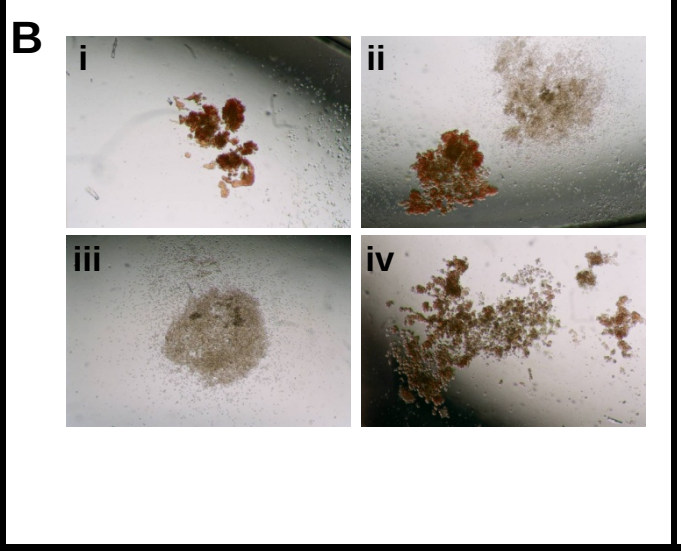
Figure 2. Reprogramming of 21-year old frozen human CB $CD34^+$ cells to iPS cells (A-H) and recovery of ECFCs (I). The phase contrast images in panels B (FCB-iPS and AP stain), E (top left image) and I were viewed using a Zeiss Axiovert 25 CFL inverted microscope with a 5x CP-ACHROMAT/0.12 NA objective. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer's software. Phase contrast images were taken with air objectives. The confocal images in panel B and E were viewed with a Zeiss Axiovert 100 LSM 510 confocal microscope using as objective a C-Apochromat (at 40x/1.2W corr.) or Plan-Apochromat (at 10x/0.45). Images were token by epifluorescence detector and transillumination detector and processed using Zeiss LSM Image Browser Version 3,5,0,376 software (all from Carl Zeiss, Jena, Germany). All the images were taken at room temperature. Cells were mounted in proLong Gold antifade mounting reagent with DAPI (Invitrogen, USA) and confocal images were taken using water immersion objectives.

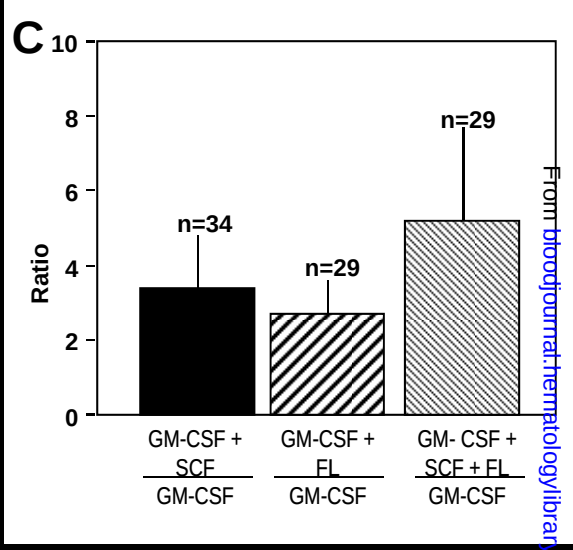
(A) Schematic representation of strategy used at IU to reprogram $CD34^+$ cells from frozen human CB. TPO, Thrombopoietin; hSCF, stem cell factor; Flt2L, fms-like tyrosine kinase 3

ligand; Y=Y-27632; VPA=valporic acid; SB=SB202190. (B) Immunocytochemistry for pluripotency markers OCT4 (Alexa 448 or 546 nm), NANOG (Alexa 488 or 564 nm), TRA-1-60 (Alexa 564 nm), SSEA4 (Alexa564 nm) and alkaline phosphatase (AP). Small panels on upper part of sub-figures show total cell content per field stained with 4,6-Diamidino-2-phenylindole (DAPI). Scale bars represent 50µm. (C) Quantitative RT-PCR analysis for expression of endogenous ES cell-marker genes OCT4, SOX2 and NANOG in hESC line H9, two iPS cell lines generated, and parental $CD34^+$ cells. Specific primers were designed to probe for 3´ untranslated region (Endo) to measure expression of the endogenous gene. Individual PCR reactions were normalized against beta actin and plotted relative to expression levels in hESC. (D) Each horizontal row of circles represents an individual sequencing reaction for a given amplicon. Open and filled circles respectively represent unmethylated and methylated CpGs dinucleotides. (E) Morphology of 10-day-embryoid body (EB) under phase contrast microscopy (upper left). Immunostaining of frozen CB (FCB)-iPS derivatives on day 10 of differentiation revealed expression of ectodermal (Nestin and Tuj1), mesodermal (alpha-SMA and Vimentin) and endodermal (AFP and Sox17) marker proteins (all with Alexa 546nm). Nuclei are stained with DAPI (blue). Scale bars represent 50 µm. (F) Teratomas derived from immunodeficient mice injected with FCB-iPS cells shows tissues representing all 3 embryonic germ layers, including secretory epithelium (ectoderm), muscle fibers (mesoderm), and gut epithelium (endoderm). Samples from the teratomas were paraffin-embedded and serially sectioned (5 µm) using a microtome (Leica Microsystems, Wetzlar, Germany). To analyze the three germ-layer lineage cells derived from injected FCB-iPS cells in the teratomas, sectioned slides were histologically examined by hematoxylin and eosin for the gut epithelium and special stains as follows: PAS stain for the secretory epithelium, and Masson's trichrome stain for muscle fibers.

Images were taken with a Nikon Eclipse 50i (Nikon Sankei, Tokyo, Japan) equipped with a digital camera (Infinty 2 Megapixel, Lumenera Corp.) at a magnification of Plan Fluor 10x0.30 and analyzed using the i-solution image analysis program (iMT i-Solution Inc., Korea). Teratoma images were taken with air objectives. Results in B-F were from studies performed at IU. Results in G, H were from another 21 year cryopreserved CB sample sent to, thawed and separately reprogrammed at Johns Hopkins Medical Center[12] after isolation of $CD34^+$ cells. For studies in G and H, a Nikon Eclipse TE2000-U (Nikon USA, Melville, NY) microscope was used, with ELWD Plan Fluor, NA:0.45 at 25°C. For G, fixed slides were used with H & E staining, and for H, PBS plus 0.1% BSA was used with Alexa 555 and DAPI. Images were taken with Imaging Micropublisher 5.0 (Q Imaging, Surrey, BC, Canada) camera with Q Capture Version 3.1.2 (Q Imaging) software. Markers for teratoma formation in vivo (G) and for differentiation of EBs from reprogrammed iPS cells in vitro (H). (I) Representative ECFC colony from MNCs isolated from 21 year CB defrosts and cultured for 18 days (left) compared with colony from freshly obtained CB cultured for 12 days. Arrows denote size of colonies. Note that even after 18 days culture of frozen CB derived colonies, the colony size is not as large as that of colony from fresh CB at 12 days of culture.



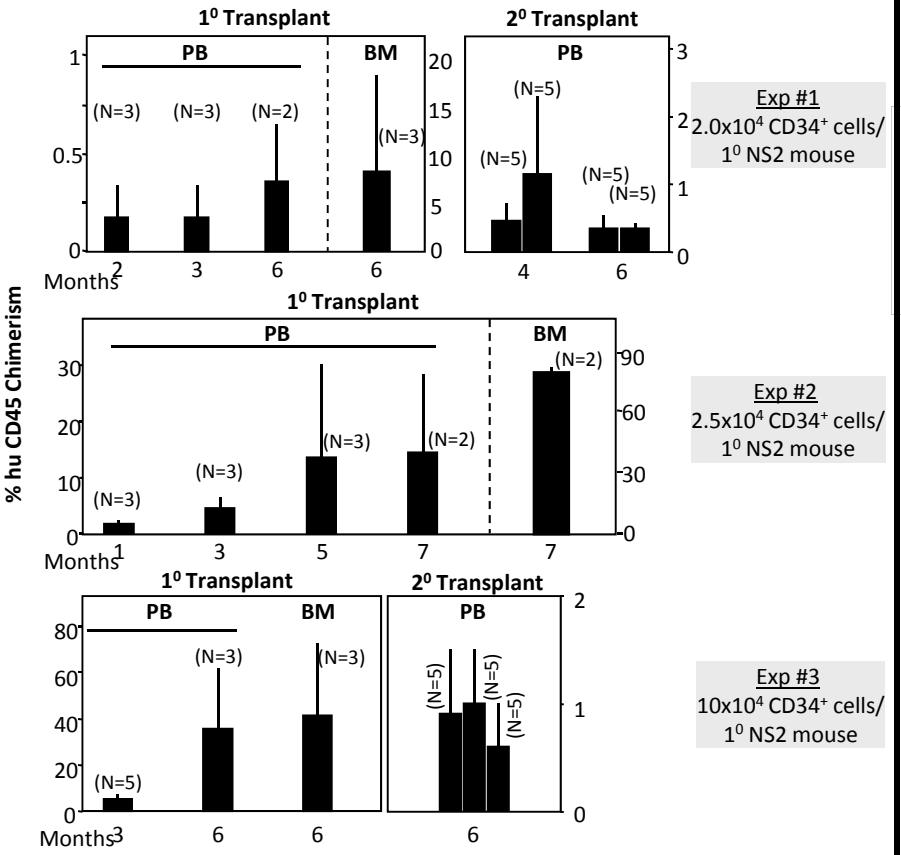




D

	% Replates with at least one colony
1⁰ Colony	**Range**
CFU-GEMM (Epo, SCF, IL-3, GM-CSF)	67-94%
CFU-GM/M (Epo, SCF, IL-3, GM-CSF)	81-93%
CFU-GM/M (GM-CSF, SCF, FL)	72-100%
	# 2⁰ Colonies/ 1⁰ Colony Replated
CFU-GEMM (Epo, SCF, IL-3, GM-CSF)	11-22
CFU-GM/M (Epo, SCF, IL-3, GM-CSF)	6-13
CFU-GM/M (GM-CSF, SCF, FL)	23-49

Primary colonies grew in presence of Epo, SCF, IL-3, GM-CSF or GM-CSF, SCF, FL. Single colonies replated in presence of Epo, SCF, IL-3, GM-CSF



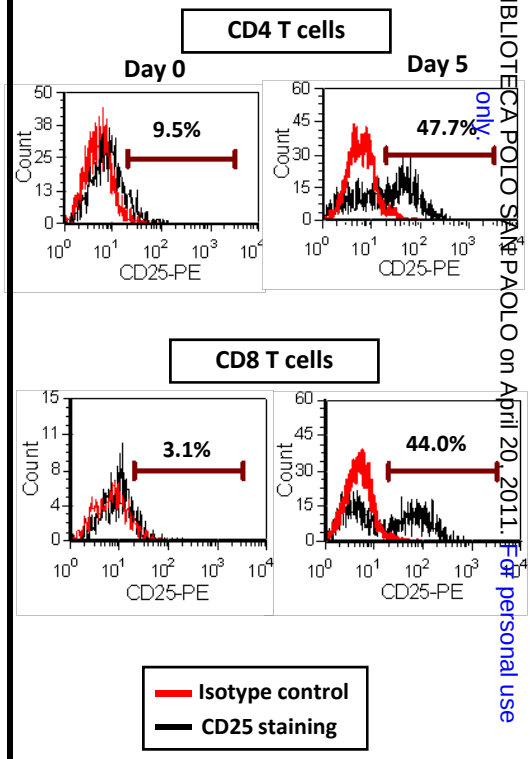


Figure 1

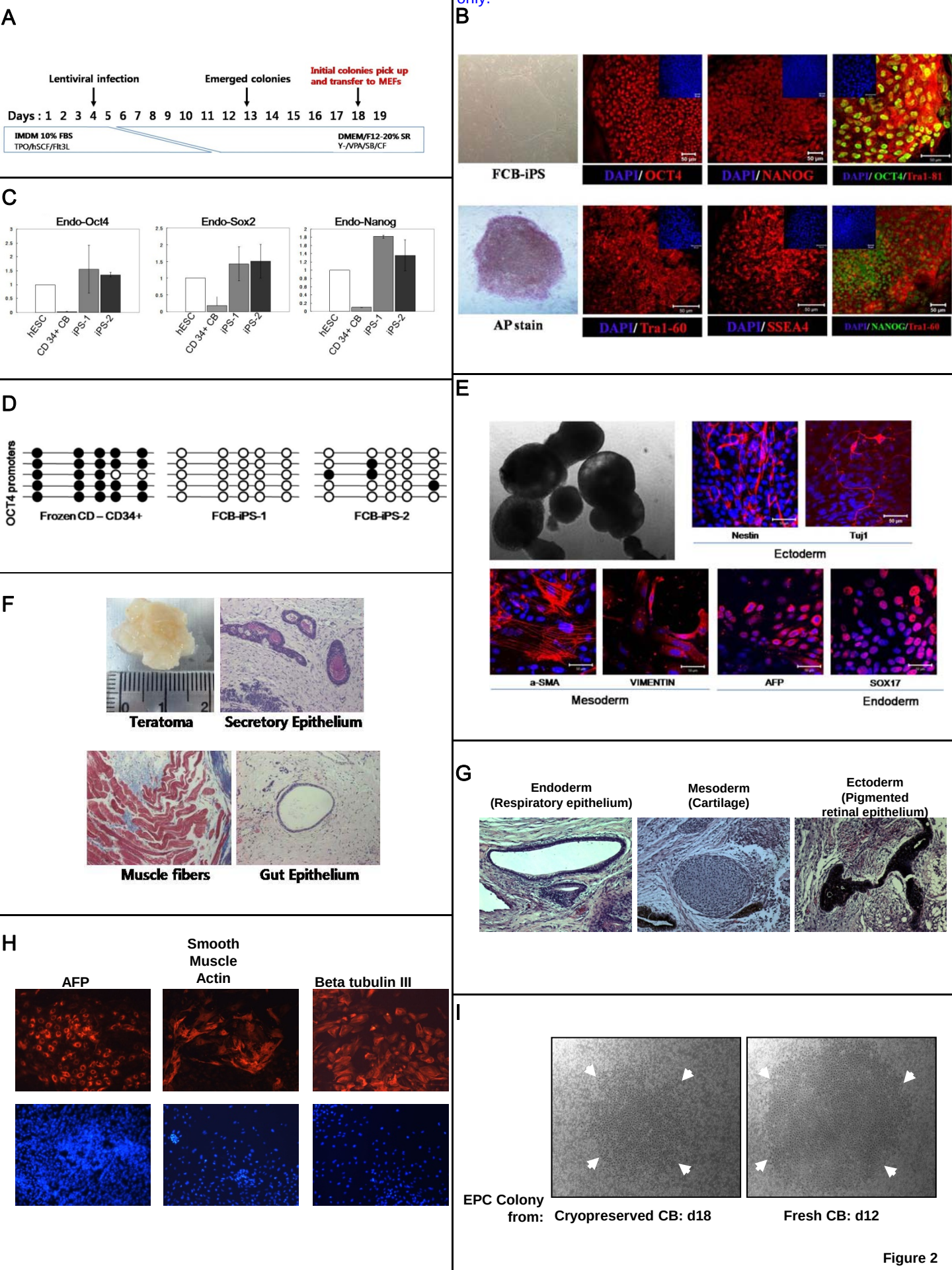

A
Lentiviral infection
Emerged colonies
Initial colonies pick up and transfer to MEFs
Days : 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
IMDM 10% FBS
TPO/hSCF/Flt3L
DMEM/F12-20% SR
Y-/VPA/SB/CF
B
FCB-iPS
DAPI/ OCT4
DAPI/ NANOG
DAPI/ OCT4/Tra1-81
AP stain
DAPI/ Tra1-60
DAPI/ SSEA4
DAPI/ NANOG/Tra1-60
C
Endo-Oct4
Endo-Sox2
Endo-Nanog
hESC
CD 34+ CB
iPS-1
iPS-2
D
OCT4 promoters
Frozen CD – CD34+
FCB-iPS-1
FCB-iPS-2
E
Nestin
Tuj1
Ectoderm
a-SMA
VIMENTIN
AFP
SOX17
Mesoderm
Endoderm
F
Teratoma
Secretory Epithelium
Muscle fibers
Gut Epithelium
G
Endoderm
(Respiratory epithelium)
Mesoderm
(Cartilage)
Ectoderm
(Pigmented
retinal epithelium)
H
AFP
Smooth
Muscle
Actin
Beta tubulin III
I
EPC Colony
from:
Cryopreserved CB: d18
Fresh CB: d12

Figure 2