中文摘要

關鍵詞：造血幹原細胞、體外增殖、臍帶血、紅血球、人類間質幹細胞

近年來幹原細胞生理特性探討及臨床應用的潛能受到廣泛重視。幹原細胞主要受到外在因子及內在因子的影響共同決定細胞的命運。血球幹原細胞因為可分化成各種不同功能的血球系，因此它具備多重複雜的分化調節機制。如何控制細胞滯留於未分化狀態，以及特定細胞種類的分化是經由調控某些特定分化基因的表現，目前尚未非常清楚。本計畫旨在有效率地分離純化造血幹原細胞並冷凍保存，提供幹原細胞臨床應用或基礎研究。我們延續對（1）造血幹細胞之分離及針對幹細胞往紅血球分化相關基因進行分子特性探討。（2）藉由二維電泳分析比對紅血球分化早中晚之蛋白圖譜得知與分化相關之蛋白種類。（3）利用免疫缺陷小鼠將組織間質幹細胞與造血幹細胞進行骨髓移植比較間質幹細胞之來源與輔助造血之功能。我們將少量早期紅血球母細胞之 mRNA 進行放大數千倍反應以供 cDNA array 分析。針對早期胚胎肝臟及新生兒臍血與成人週邊血之造血幹細胞在紅血球生長分化上之差異比較結果顯示 CD34+HSP/C 幹細胞具有發育階段特異性及功能決定性，此差異不影響單一血球系成熟的路徑。另外，為了探討基質細胞與造血系統微環境之互動影響，我們也分離自不同組織分離人類間質幹細胞並進行體外分化，探討其多重分化潛能。初步觀察到不同來源有分化為間質組織功能細胞的現象。

具體而言，我們在本計畫年度共完成（一）利用所建立之體外紅血球生成的模式，經由基因晶片分析及二維電泳分析，探討紅血球成熟過程之早、中、晚期基因及蛋白表達之分子機制。（二）我們觀察比較得知人體組織基質細胞之非同質性、組織功能性。（三）增進造血幹細胞之增殖及體外維護效率。為得知是否能協助造血幹原細胞，我們也分離具基質細胞潛能之人類間質幹細胞 PLA，與造血幹細胞同時給予體內免疫缺陷 (NOD/SCID) 之老鼠，比較骨髓及脂肪取得之間質幹細胞分化潛能差異以及不同來源提供造血微環境 (microenviroment)的能力之相關性。

Progress Report on NSC92-2318-B-038-001-M51 (8/2003-7/2004): Molecular and Cellular Characterization of Hematopoietic Stem Cells (HSC) and Progenitor Cells (PGC)

Abstract

KEY WORDS: CD34+ Hematopoietic stem/progenitor cells, umbilical cord blood,

erythroblast, human processed lipoaspirate

In recent years, the multi-potentials of CD34+ hematopoietic stem/progenitor cells (HS/PC) on broad clinical applications are widely investigated. Characterization of HS/PCs has therefore been the key for understanding how normal hematopoiesis precisely regulated at molecular level. Because of the multiple-lineage commitment capacity of HS/PCs is decided both by extrinsic (environmental) and intrinsic (intracellular) factors. However, due to the limited number and the lack of unique cell marker of HS/PCs have hampered the progress of many studies. In the previous budget years, we have established a solid phase mRNA amplification, single cell culture, and single cell RT-PCR technologies that allowing us to continue explore this invitro study. In this budget year, we have continue (i) to characterize the CD34+ HS/PC subpopulation, identifying structural and functional changes in lineage specific erythropoietic differentiation of CD34+ HS/PCs and (ii) to examine the proteomic variation of the early- and late- erythroblasts by two dimensional electrophoretic mapping (2DE) analysis. (iii) to identify the cross-talk between stromal cells and HSCs that supporting normal hematopoietic reconstitution, we have conducted a comparative analysis of MSC and PLA that influencing the CD34+ HS/PCs transfusion efficiency in NOD/SCID mice. We have focused in comparative molecular characterizations of the CD34+ HS/PC population in fetus, new born and adult in terms of identifying their characteristic differences in lineage specific erythropoietic differentiation. We have also achieved a thousand folds in amplification of mRNA of early erythroblasts for cDNA microarray and subtraction analyses. To understand the supporting role of stromal cells that supporting normal hematopoietic reconstitution and ex vivo expansion we have isolate and primary culture of several tissue mesenchymal stem/progenitor cells and characterizing their potential heterogeneity. A comparison of MSC and PLA that influencing the CD34+ HS/PCs transfusion efficiency in an in vivo NOD/SCID mice study has been carried out and confirming the tissue specified role of the somatic MSCs.

In summary, we have accomplished the proposed studies in this budget year including I) to identify the developmental stage dependent erythropoietic gene expression regulatory differences. II) to identify the molecular and functional heterogeneity of human tissue mesenchymal stem/progenitor cells. III) to improve the in vitro maintenance and expansion of primitive hematopoietic stem cells. To understand the cross-talk between stromal cells and HSCs that supporting normal hematopoietic reconstitution, we have isolate and primary culture of processed lipoaspirate (PLA) and characterizing their differentiation potentials. A comparison of MSC and PLA that confirming the supporting the CD34+ HS/PCs transfusion efficiency by MSCs in NOD/SCID mice has been made.

Results and Discussions

Confirmation of Bone Marrow MSC for better CD34+ HS/PCs transfusion efficiency in NOD/SCID mice

Recent studies have shown the association of mesenchymal stem cells (MSC) with HS/PC may increase the HS/PC transplantational engraftment efficiency (Angelopoulou, M.K. 2001). Studies of Processed lipoaspirate (PLA) have shown these cells are mesenchymal origin with similar differentiation potential as MSC does. Therefore, we have co-transfused the isolated bone marraw MSC and fat tissue PLA with human cord blood CD34+ HS/PCs to the 350cRad irradiated NOD/SCID mice for a comparative *in vivo* study (Tab II). Our data indicated that the molecular and

functional heterogeneity of human tissue mesenchymal stem/progenitor cells may explain the ideal marrow homing microenvironment for hematopoietic stem/progenitor cells as reported at Eleventh Symposium on Recent Advendes in Cellular and Molecular Biology (Isolation and Characterization of Human Adipose Tissue Mesenchyam lStem/Progenitor Cells (Fat-MS/PC): A Comparative Study on Fat-MS/PCs and Bone Marrow-MS/PCs.

An In Vitro Study on the Developmental Stage Dependence of Hematopoietic Erythroid Gene Expression Regulations

In this budget year, we have compared a series of *in vitro* erythroid differentiation cell culture of CD34+ HS/PCs derived from fetus, new born and adult origins, under the stimulation of erythropoietin.

This culure system primary expand the CD34+ HSP/Cs for seven days to generate relatively homogeneous erythoid progenitors and then direct the erythroid specific CD36+ cells in culture toward erythrocyte commitment differentiation that allowing the culture cell quantity enough for a series of analyses (Fig 1). Specifically, we focused on molecular characterization of the lineage-committed progenitor cells by colony forming, flow-cytometry, histochemistry, RT-PCR, micro-array analysis and two-dimensional gel electrophoresis under stimulation of hematopoietic cytokines erythropoietin (Epo) and stem cell factor (SCF) (Fig 2~4). SCF and Epo were essential for primitive/ difintive stage erythroid differentiation. They also promoted cell proliferation relative effective by early (SCF) and late (Epo) developmental stages during erythropoiesis. We have identified novel gene expression profiles for erythropoiesis of fetus or adult type erythrocytes in early differentiation stage. We found similar genes were induced in CD36+ cells during early stage of Epo dependent erythropoiesis irrespective of their original developmental stage (Tab.I). Erythroid differentiation relatedgenes were indentified by cDNA microarray. However, other myeloid and even non-hematopoietic lineage genes were down-regulated in Epo dependent differentiation of fetus type erythrocytes, which were not observed in counterparts from latter developmental stages. RT-PCR comfirmed the high differential expressed candidates in microarray result. Our result indicated developmental discrenceny of erythroid progenitors (CD36+ cell) derived from CD34+ HSP/Cs (Fig.5). These data also demonstrated that the developmental regulatory of globin synthesis can be shown in the RBC formation cell culture model (Fig.3).

Two dimensional electrophoresis (2-DE) of early- and late- erythroblasts

To evaluate the cellular protein changes during erythropoiesis, we compared the protein expression profiles of CD36+ cells before and after stimulation by Epo using gel electrophoresis analysis (Fig 6, Tab III). Compare to public protein database that related to hematopoiesis, these proteins can be classified into cell proliferation, differentaion, cytokines, transcription factors, chemokines and their receptors. We observed a detectable, major expression difference of structure proteins been regulated during erythroid differentiation.

Establishment of Stroma Based Ex vivo Expansion of Primitive Hematopoietic Stem Cells

Previously studies by us and others have shown that ex vivo expansion of CD34+ HS/PCs is needed a stroma environment for maintaining HSC in the undifferentiation state. In this year of study we have isolated several MSCs and characterized their phenotypical specification. By gene expression profile analysis and cell culture in serum

and serum free culture media selection we have established several MSCs for supporting CD34+ HS/PCs expansion in the co-culture systems. The expansion efficiency in both cd34+ cell numbers and their colony formation potentials are shown in Table IV, and these CD34+ HSCs are capable in forming colbostones in the long term cell culture (LTC-IC) system indicating that the expanded CD34+ by our method includes the expanded primitive HSCs (Fig.7). An in vivo NOD/SCID mice transplantation assay are needed for verifying this suggestion.

Self-evaluation

In this budget year have accomplished the proposed study I) to identify the developmental stage dependent erythropoietic gene expression regulatory differences. II) to identify the molecular and functional heterogeneity of human tissue mesenchymal stem/progenitor cells. III) to improve the in vitro maintenance and expansion of primitive hematopoietic stem cells. To understand the cross-talk between stromal cells and HSCs that supporting normal hematopoietic reconstitution, we have isolate and primary culture of processed lipoaspirate (PLA) and characterizing their differentiation potentials. A comparison of MSC and PLA that confirming the supporting the CD34+ HS/PCs transfusion efficiency by MSCs in NOD/SCID mice has been made

We have achieved several progresses on better insights into the structure and function of HS/PC and their association with stromal environment in last two years. The reproducible cell preparation and characterization procedures have been well established. We have also set up a definitive RBC maturation model for in vitro studies erythropoiesis mechanism. Although we have well-defined systems for stem cell analysis, further evidences are required for genome and proteomic studies, like the putative candidate genetic construction and peptides identification. In the coming year, we will finish c-DNA subtraction and high-resolution 2-Dimensional electrophoresis to identify and verify novel genes and proteins involved in regulating the hematopoiesis during the course of in vitro cell culture. To understand the regulatory pathway during hematopoiesis more functional studies are required. We also attempt to use single cell sorting technique to isolate unique subpopulation of HSCs for further studies but the technical issue is still under the investigation. Lineage specific differentiation culture seeded from single cell origin will be help to resolve puzzle of stem cell heterogeneity. The success of global genetic and proteomic studies in the last year may enhance our ability to characterize the maturation of hematopoietic cells at the molecular level and facilitate functional studies on the lineage specific novel genes.

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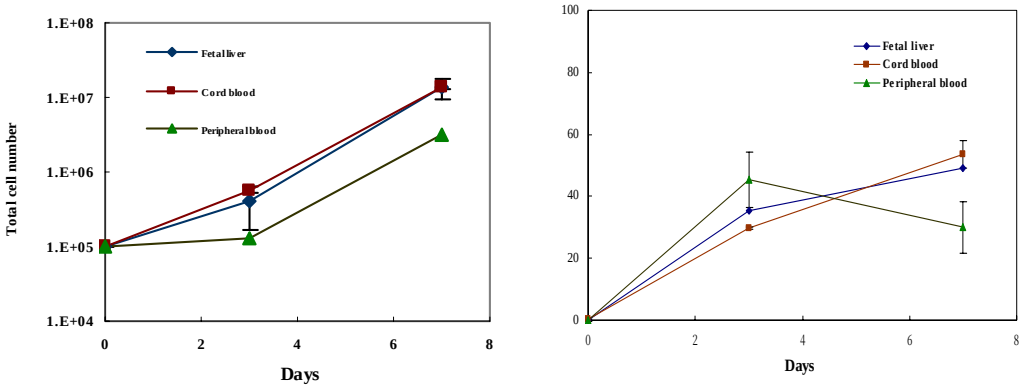

Fig 1. Cell Expansion of Hematopoietic and Erythropoietic Progenitors by Hematopoietic Factors rhSCF, rhTPO, rhIL-3, rhIL-6 and Flt-3 Ligand. CD34+ HS/PCs and CD36+ erythroid progenitors were isolated from MNCs of fetal liver, cord blood and peripheral blood by magnetic cell sorting method. Alive cells were counted by trypan blue staining.

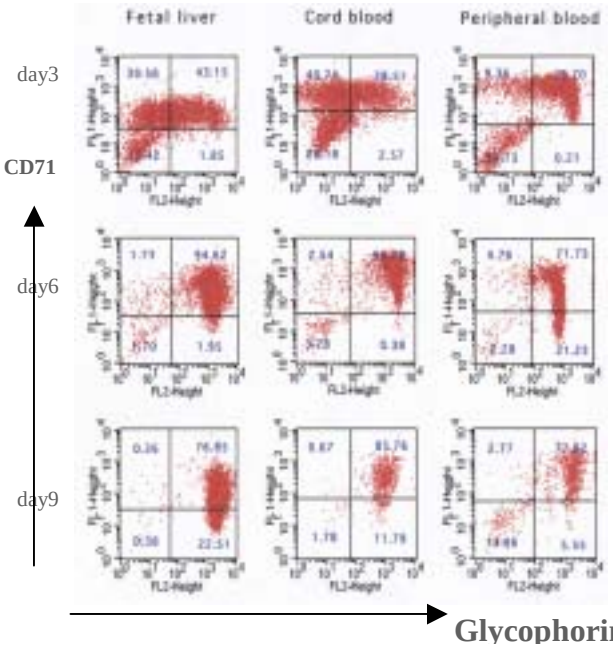

Fig 2. Surface antigen expansion of EPO stimulated differentiating erythroid cells. Cells were stained with florecece conjugated specific antibodies CD71 and Glycophorin A and were analyzed by flow cytometry at day3, 6 and 9 of differentiation culture.

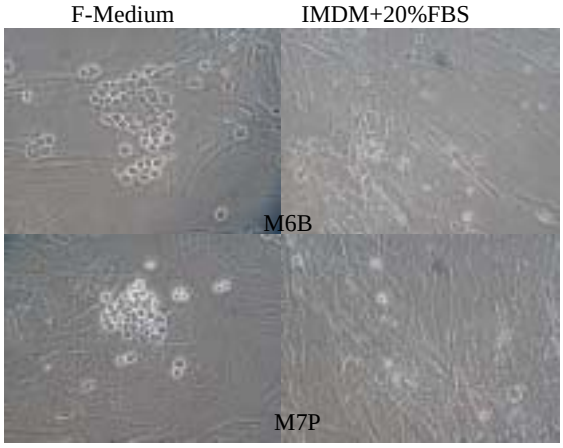

Fig 7. Cobblestone formation of CD34+ HSP/Cs expansion culture on M6B or M7P.

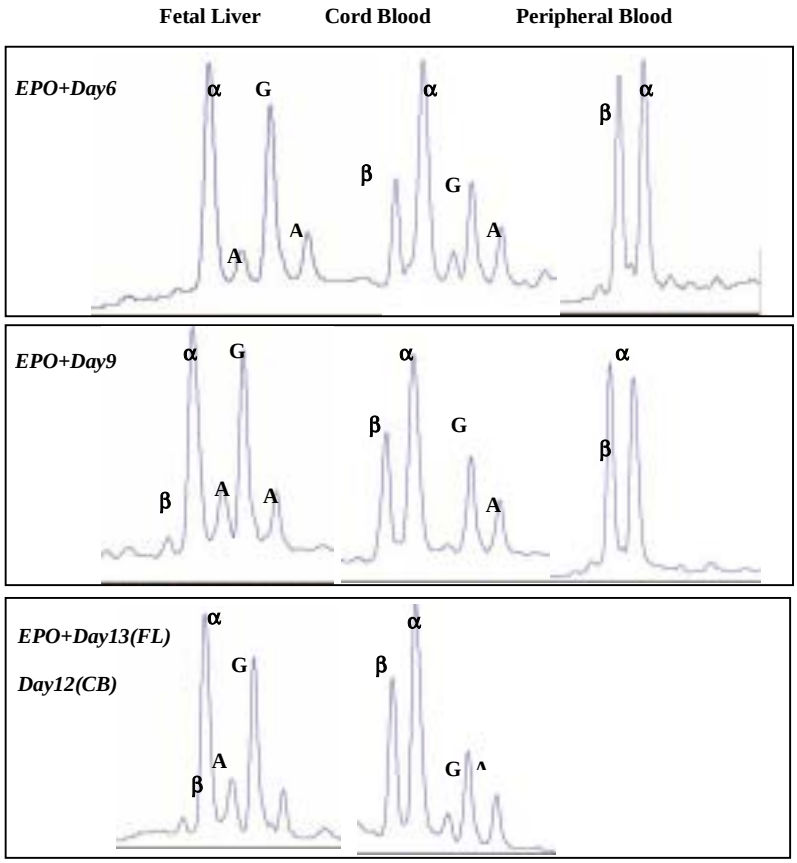

Fig 3. HPLC analysis of hemoglobin expressions of maturing erythrocytes derived from different developmental stages.

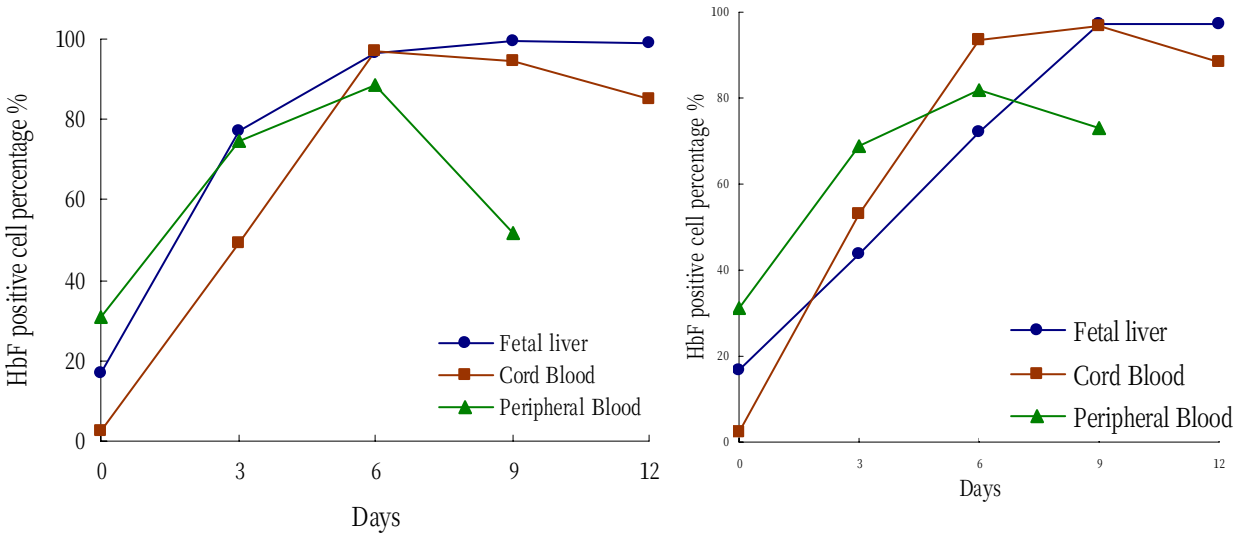

Fig 4. Increase of HbF population during Epo dependent erythropoiesis of CD36+ progenitors. F hemoglobin is expressed early in blast stage and used here as a tracer for their functional maturation progress.

Tab I. cDNA microarray result of genes upregulated during erythropoiesis of CD36+ progenitors derived from FL and CB (GPA+ vs GPA-) (A) or genes up-regulated in fetal liver CD36+ progenitors but down regulated in cells from cord blood (B).

(A)

Gene Name	Accession No.
Human cystic fibrosis antigen mRNA, complete cds.	**M26311**
CD74 antigen (invariant polypeptide of major histocompatibility)	**AL543515**
vimentin	**AL046515**
interleukin 18 (interferon-gamma-inducing factor)	**BC007007**
interleukin 8	**BG777366**
pyruvate kinase, muscle	**BF690275**
interleukin 1, beta	**AA577318**
thymosin, beta 10	**AV717021**
Human monocyte-derived neutrophil-activating protein (MONAP)	**M26383**
platelet factor 4	**AI078445**
glia maturation factor, gamma	**AA279067**
S100 calcium-binding protein, beta (neural)	**AV728996**
coronin, actin-binding protein, 1A	**BG758313**
ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	**L35594**
CCAAT/enhancer binding protein (C/EBP), alpha	**BG481789**
Human NAD+-dependent 15 hydroxyprostaglandin dehydrogenase	**L76465**
interferon, gamma-inducible protein 16	**BG434340**

(B)

Gene Name	Accession No.
Human red cell anion exchanger (EPB3, AE1, Band 3) gene	X77738
Human maltase-glucoamylase mRNA, complete cds.	AF016833
Human glycophorin A, MN-types (GYPA) mRNA, complete cds.	L31860
hemoglobin, beta	BG622572
peroxiredoxin 3	BI862079
Human arachidonate 13-lipoxygenase mRNA, complete cds.	M62982
erythropoietin receptor	S45332
hemoglobin, gamma G.	AI133196
hemoglobin, delta	BG943559
Human, hydroxymethylbilane synthase, clone MGC:8561.	BC000520
Human, tubulin, gamma 1, clone MGC:1593.	BC000619
Human erythroid alpha-spectrin (SPTA2) mRNA, complete cds.	M61877
spectrin, alpha, erythrocytic 1 (elliptocytosis 3)	AA703344
Rhesus blood group-associated glycoprotein	AU121299
nuclear factor (erythroid-derived 2), 46kD	S77763
Human, ornithine aminotransferase (gyrate atrophy).	BC000964
ESTs, Highly similar to klf2 krupple-like transcription factor.	AI040588
Human metallothionein (MT)I-F gene, complete cds.	M13003
microsomal glutathione S-transferase 4	BF792818
basigin (OK blood group)	AU120559
Human, hydroxyacyl glutathione hydrolase, clone MGC:3590.	BC002627

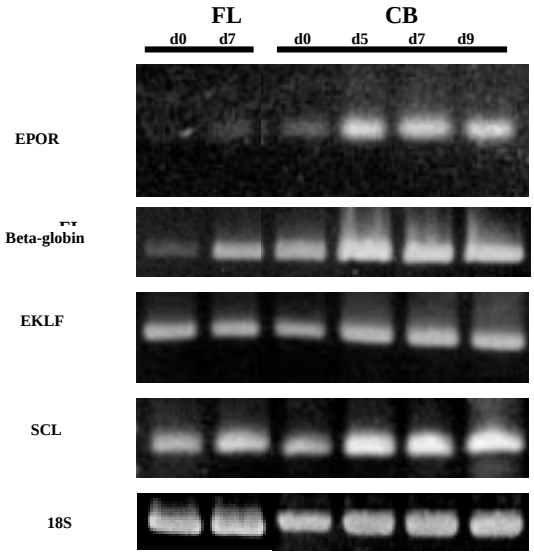

Fig 5. RT-PCR result of erythroid differentiation related genes during culture course.

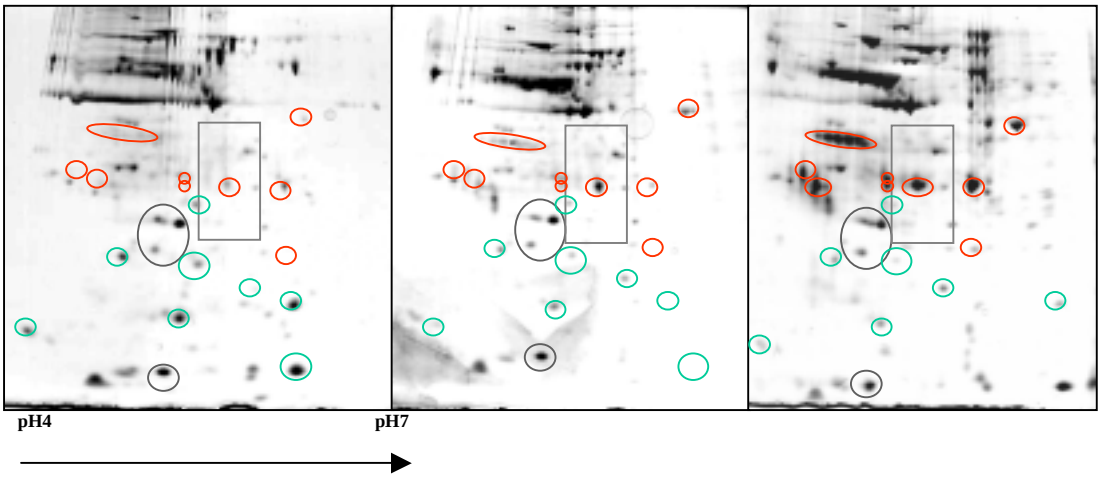

Fig 6. Mini-2-DE of differentiating erythroid cells at 2nd phase culture day 0, 3 and 6. 20μg protein from 2nd phase cultured CD36+ cells at day0, 3 and 6 was loaded for 2-DE analysis, respectively. We focused on evaluating proteins been regulated during early erythropoiesis in pH range of 4 to 7 Gels are scanned and a criteria of over 2 folds differential expressed spots are selected. The selected proteins include unique, positively and negatively regulated ones.

Tab II Hematopoietic reconstitution of NOD/SCID mice by cotransplantational of human CD34+ HS/PCs with BM-MS/PCs or PLA-MS/PCs.

Injected cell population	cells injected per mouse	positive mice/viable mice	% of CD45+ cells
CD34++MNCs	5X104+106	2/2	8.82-31.89
CD34++MSCs+MNCs	5X104+5X104+9.5X105	1/2	14.12
CD34++PLAs+MNCs	5X104+5X104+9.5X105	0/0	ND
CD34++PLAs+MNCs	5X103+5X104+9.5X105	0/0	ND
NOD/SCID mice were gamma ray subleathal irradiated(300cRad) prio to the co-transplantation cell infusions.			

Tab. III Candidate proteins participated in erythroid differentiation. Protein spots identified differential expressed in day3 or day6 compared to day0 or day3 were marked.

Category	0/3	3/6	Index
Growth Factor		√	Growth/differentiation factor 2 precursor (BMP-9).
Anti-Oxidant		√	Glutathione S-transferase Mu 2
		√	Glutathione transferase omega 1
	√		Superoxide dismutase SOD1.
Corticoid Responsive Genes	√		Tumor necrosis factor receptor superfamily member 18 precursor
Differentiation	√	√	HLA class II histocompatibility antigen, DR alpha chain precursor.
		√	HLA class II histocompatibility antigen, DP alpha chain precursor
$CD34^+$ Expression Gen	√		Integral membrane protein *(Potential)*.
	√	√	Protein C3orf4 (Membrane protein GENX-3745)
		√	Hematopoietic stem/progenitor cells expression protein
		√	Vacuolar sorting protein 4b
Transcription Factor	√	√	Zinc finger protein 46
		√	Catechol O-methyltransferase
Signal Regulator/Adap	√		Leukemia associated protein 1.
	√		Rho GDP-dissociation inhibitor 2
		√	GRB2-related adaptor protein.
		√	GRB2-related adaptor protein 2 (GADS protein)
Erythropoietic Genes	√		Cornichon homolog. Integral membrane protein *(Potential)*.
	√		Golgi-associated MP1 adapter protein.
	√		Tubulin-specific chaperone A
	√	√	Erythropoietin receptor (EPO-R).
		√	Antioxidant protein 2
		√	Erythrocyte tropomodulin (E-Tmod)
			Rhesus blood group-associated glycoprotein (RH50A)
		√	GDP-fucose synthetase (FX protein)
		√	Alpha adducin (Erythrocyte adducin alpha subunit)
Cell Cycle		√	Cyclin-dependent kinase 4 inhibitor B (p14-INK4b)
Cell Structure	√		Beta-2-microglobulin
	√	√	Tubulin-specific chaperone B
		√	Actin-like protein 2
		√	Beta-2-microglobulin precursor (HDCMA22P).
		√	Microtubule-associated protein RP/EB family member 1
Apoptosis		√	Apoptosis regulator BAX, membrane isoform alpha.

Tab. IV Mesenchymal stroma support on hematopoietic stem cells expansion in vitro. Cord blood CD34+ cells were cultured with or without feeder layers for 7 days in the presence of cytokines. Colony number includes all myeloid derived lineages. M5B, M7P and M5C were derived from different human tissues, except M5S from mouse.

	M5B(%)	M7P(%)	M5C(%)	M5S(%)	CD34+only(%)
folds increased of CD34+ cells	10.20	5.30	4.45	9.80	0.22
folds increased of CD34+ CD38-cells	12.07	8.49	21.60	41.12	0.16
colony formation assay	151	186	510	124	85