ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

A Mixture of Mesenchymal Stem Cells from Perinatal Sources Supports Consistent *Ex-vivo* $CD34^+$ Hematopoietic Stem Cell Expansion

[*1]Sushilkumar Ramdasi, [2]Chandra Viswanathan, [3]Rohit Kulkarni, [4]Abhijit Bopardikar, [5]Raghwendra Kumar, [6]Pradeep Somalapur, [7]Poonam Pandey

Research & Development Team
ReeLabs Private Limited. Andheri West, Mumbai, India, 400053

Abstract: **Umbilical cord blood transplantation has limitations due to lesser number of Hematopoietic Stem Cells (HSCs) in the graft, needed for efficient hematopoietic reconstitution. Besides that, problems in getting an appropriate HLA match and incidence of severe GvHD are inherent issues. *Ex-vivo* $CD34^+$ HSC expansion is one of the key strategies to increase HSC numbers to meet the recommended dosage requirement. Recent reports have demonstrated the plasticity of $CD34^+$ HSCs that helps differentiate them into various cell types including renal, cardiac, liver, neural, mesenchymal and muscle cells. This makes $CD34^+$ cells yet another novel cell type for regular use in regenerative medicine applications.**

Objective: The objective of this research was to establish a cost effective *ex-vivo* expansion of HSCs using perinatal MSCs as a feeder layer while maintaining stemness and functionality.

Method: We used a mixture of Mesenchymal Stem Cells derived from three perinatal sources (Umbilical cord tissue, placenta and amniotic sac) as a feeder layer for *Ex-vivo* expansion of $CD34^+$ HSCs pooled from multiple cord blood units. The media used contained different concentrations of cytokines. A mix of different MSCs release abundant growth factors and cytokines much required for the maintenance of phenotype and functionality of $CD34^+$ HSCs during expansion.

Results: We obtained average 30 fold expansion of $CD34^+$ HSCs. It was a cost effective process. The functional characterization confirmed the stemness of the expanded cells.

Conclusion: MSCs from perinatal sources is a good option as a feeder layer for the *Ex-vivo* expansion of $CD34^+$ HSCs. This source of MSC is ontogenetically naive and can produce more amounts of cytokines for sustainable HSC expansion.

Keywords: ***Ex-vivo*** **expansion, $CD34^+$ HSCs, Mesenchymal Stem Cells, Perinatal sources, Regenerative Medicine, Co-culture.**

I. INTRODUCTION

Related and un-related umbilical cord blood stem cell transplantation is an established therapy for treatment of hematological malignancies with or without a complete HLA match [1][2][3]. The disorders treatable with cord blood stem cells include Leukemia, Sickle Cell Anemia, Thalassemia and certain autoimmune diseases [4]. Additionally, there is an increasing evidence that hematopoietic stem cells, with their characteristic $CD34^+$ positivity, play a crucial role even in the regenerative process in various ischemic pathologies, support transdifferentiation into cells such as hepatocytes cardiomyocytes and respiratory epithelial cells[5] [6], [7], [8] in animal models. The role played by $CD34^+$ cells in neurological recovery has been established in animal models of stroke. It has been noted that neurogenesis and angiogenesis are enhanced after administration of $CD34^+$ cells to such models [9]. Hematopoietic stem cells are also believed to have a role in vasculogenesis in ischemic kidneys [10]. Many pre-clinical studies highlighted the significance

of $CD34^+$ HSCs in restoring functions of ischemic myocardium by improving blood flow [5][11]. Numerous clinical studies in humans have been encouraging with respect to angiogenic potential of HSCs in myocardial ischemia as compared to traditional therapy regimens [12]. It is amply evident that the mobilization of peripheral blood $CD34^+$ cells results in faster neurological and functional recovery in patients with stroke [13].

To address the various above unmet clinical needs, the umbilical cord blood derived $CD34^+$ HSCs will need to be expanded *in vitro* to meet the requisite dosage requirements [3][4]. *Ex-vivo* expansion is equally important and necessary for application of $CD34^+$ HSCs in regenerative medicine applications, since umbilical cord blood is not an abundant source of HSCs. For this reason, expanding a single umbilical cord blood graft for use in regenerative medicine application turns out to be financially non feasible. It involves use of expensive growth factors, magnetic purification consumables, leaving us with poor yields. However, optimization of pre-expansion HSC numbers by pooling multiple cord blood units seems more cost effective. This pooling could be useful in regenerative medicine applications as histocompatibility is not a requirement [14].

Umbilical cord blood $CD34^+$ HSCs are more naive than those derived from adult sources [14]. Also cord blood is less cytotoxic than bone marrow or peripheral blood as there are less immune cells in the cord blood compared to their counterparts [15][16][12]. These HSCs have longer telomeres and greater proliferation potentials that make it an attractive source of stem cells [17][18][19].

In the present study, we have looked at all aspects of expanding $CD34^+$ HSCs from pooled umbilical cord blood units from different donors, using a feeder layer, having a mixture of Mesenchymal Stem Cells (MSCs) derived from three different perinatal sources viz. cord tissue, placenta and amniotic sac. We attempted to establish a cost effective processes that will make it worthy of being considered as a regular treatment offering.

II. MATERIALS AND METHODS

Umbilical cord blood samples were collected immediately after delivery, following universally accepted procedures and after due approvals from the Institutional Ethics committee [IEC]. The expectant mothers were counseled for benefits, discomforts, risks associated with the collection and a detailed medical history was obtained. Maternal blood samples were also collected to test for freedom from infectious disease agents, following appropriate guidelines and laws in force in this region. The maternal samples and the cord blood were transported to the processing laboratory between 15 to 22^0C in a specially made validated box; cord blood units were processed within 48 hours of collection.

A. Collection of Umbilical Cord Tissue, Placenta and Amnion

The cord tissue, the placenta and the amniotic sac were collected after the delivery following a standard procedure. Approximately two pieces of 10 cm each of the cord tissue were cut and transported in shipping media containing phosphate buffer saline (PBS) containing antibiotics. The whole placenta along with amniotic sac was shipped in a container having PBS with antibiotics. All the biological tissues were shipped in a validated temperature controlled box between 15 to 22^0C. Tissues were processed within 48 hours of collection.

B. Isolation of Mesenchymal Stem Cells (MSCs) from Cord Tissue, Placenta and Amnion

MSCs were isolated from the cord tissues by following the in-house validated protocols [20]. Briefly, the cord tissue was cleaned with PBS (Cellclone, Genetix, India) pre-treated with antibiotics. The ends of cord tissue were cut and thrown away. About 2 cms cord tissue pieces were selected and blood clots if any were removed from this segment. Smaller explants were dissected cross sectionally and placed into tissue culture-treated dishes in an expansion media containing DMEM/F12 (Gibco, USA) with 10 % serum (Gibco, USA) and 2 ng/ml bFGF (Invitrogen, USA). The cells were allowed to grow out of the explants and further expanded into monolayer cultures at 37^0C and 5% CO_2. The media was changed every second day and cells were sub-cultured upon confluence and characterized at every passage.

MSCs from the placental tissue were derived by enzymatic digestion using 0.4% collagenase type IV (Gibco, USA) and 0.01% DNase (Roche, Germany). This mixture was incubated at 37^0C for about an hour to achieve complete digestion. The suspension so obtained was washed twice with PBS (Cellclone, Genetix, India) and seeded into a tissue culture treated flask in expansion media containing DMEM/F12 (Gibco, USA) with 10 % serum (Gibco, USA) and 2 ng/ml bFGF (Invitrogen, USA). The cells were allowed to grow and further expanded in a monolayer culture at 37^0C and 5% CO_2. The media was changed every second day and cells were sub-cultured upon confluence and characterized at every passage.

The amniotic tissue was extracted from the fetal side to avoid maternal cell contamination. The tissue was mechanically fragmented and the resultant tissue pieces were washed with PBS (Cellclone, Genetix, India) multiple times. The tissue pieces were then subjected to enzymatic digestion in two stages. The first stage was to remove epithelial amniotic cells by incubation with recombinant Trypsin (Trypsol-AOF®, Biogenomics, India) at 37^0C for 5 minutes. The second stage was to make a single cell suspension in which collagenase type IV (Gibco, USA) was used at 37^0C for 5 minutes. The enzyme was then inactivated by serum. The suspension so obtained was then filtered and washed with PBS (Cellclone, Genetix, India). The cell suspension was seeded into tissue culture treated flask in expansion media containing DMEM/F12 (Gibco, USA) with 10 % serum (Gibco, USA) and 2 ng/ml bFGF (Invitrogen, USA). The cells were allowed to grow and further expanded in a monolayer culture at 37^0C and 5% CO_2. The media was changed every second day and cells were sub-cultured upon confluence and characterized at every passage.

C. Co-culturing MSCs from Cord Tissue, Placenta and Amniotic Sac

The MSCs at passage zero (after primary culture) from all the three sources viz. cord tissue, placental tissue and amniotic sac were co-cultured into a tissue culture-treated flask at a ratio of 1:1:1 in a similar expansion media. The media was changed every second day and cells were sub-cultured upon confluence and characterized at every passage.

D. Umbilical Cord Blood Processing and Storage

The umbilical cord blood was processed in a GMP (Good Manufacturing Practices) compliant clean room. The collection volume of cord blood was determined and an equal volume of Hydroxy Ethyl Starch (HES) was added to the cord blood bag and after proper mixing, hanged upright for sendimenting RBCs. The settled RBCs at the bottom were removed from the bag through one of the ports and discarded. The stem cells' rich plasma left in the bag was transferred to 50 ml sterile centrifuge tubes. These tubes were centrifuged at 1500 rpm for 10 minutes to pellet down nucleated cells. The final volume of the plasma along with the nucleated cell pellet was made up to 20 ml. This cell suspension was then pre-cooled on ice packs and 5 ml of 10% cold DMSO (Dimethyl Suphoxide) was added very slowly with intermittent gentle mixing. The cells were then transferred into 5 ml cryovials. The cells were then controlled rate frozen and stored at -196^0C in vapour phase of nitrogen.

E. Characterization of Umbilical Cord Blood and Mesenchymal Stem Cells

The umbilical cord blood was subjected to pre-process and post-processes cell counts, then characterize by flow cytometry following ISHAGE guidelines for enumeration of $CD34^+$ hematopoietic stems cells and the viability [21]. The mononuclear cells (MNCs) and expanded $CD34^+$ hematopoietic stem cells were also subject to flow cytometry and viability analysis. Briefly, approximately 50,000 cells were added with FITC labelled antiCD45 and PE labelled antiCD34 antibodies (BD Pharmingen, USA). The stained cells were acquired, and about 20,000 events were analysed using Cell Quest software (BD, CA, USA), in a FACSCalibur instrument.

MSCs were characterized for cell surface markers such as CD90, CD73, CD105 and HLA-DR antibodies. Conjugated with FITC or PE or PerCp or APC (All antibodies conjugated with fluorochrome were from BD Pharmingen, USA).

F. Isolation of Mononuclear Cells (MSCs) from Frozen Umbilical Cord Blood Units

The cryopreserved stem cell enriched cord blood units stored in vapor nitrogen formed the starting material to isolate MNCs. Multiple cord blood units were used to set up a single expansion batch. The starting absolute $CD34^+$ cell count at the beginning of every batch from multiple cord blood units was kept between 10 to 15 million. The selected cord blood units were retrieved from vapor nitrogen and rapidly thawed at 37^0C with intermittent mixing as per our SOP. Upon reaching a semi-thaw state, the contents from one cord blood unit were collected into one 50 ml sterile tube. To this 25 ml thawed cord blood unit, an equal volume of thawing media (containing 4% serum in PBS and added with DNase) was added and mixed well to avoid clumping of cells. The cells in all 50 ml tubes were washed and resuspended in thawing media. The cell suspension was layered onto equal volume of Ficoll (Histopaque, Sigma, USA) for density gradient centrifugation at 400g for 20 minutes. The MNC ring from the interface was collected in another 50 ml tube. Then, MNCs from multiple cord blood units were pooled into the same 50 ml tube. The pooled MNCs were washed once with the thawing media. The cells were counted using Hemocytometer and $CD34^+$ HSC enumeration was performed using flow Cytometry.

G. Purification of CD34$^+$ Hematopoietic Stem Cells from MNCs

The CD34$^+$ hematopoietic stem cells were purified from MNCs by magnetic separation. All reagents were purchased from Miltenyi Biotech, Germany. Briefly, the pooled MNCs were pelleted out, and for every 100 million MNCs, 300 µl of MACS Buffer (PBS with 4% serum) was added and mixed well. Then, for every 100 million MNCs, 100 µl of FcR blocking reagent was added followed by addition of 100 µl of CD34 microbeads to the MNC cell suspension. The MNC cell suspension along with added reagents was incubated at 2-8^{0}C for 30 to 40 minutes in the refrigerator. After incubation, the cells were washed twice with MACS buffer and resuspended in 1 ml for MS column (for less than 2000 million cells) or in 3 ml for LS column (for cells between 2000 to 200,000 million cells) of MACS buffer on the basis of cell count. The column of appropriate capacity was pre-conditioned following manufacturer's instructions. The cell suspension was passed through the column and the positive fraction of CD34$^+$ cells was collected by a depletion strategy in a fresh sterile centrifuge tube. The CD34$^+$ cell fraction was washed with MACS buffer and resuspended in appropriate media. The cells were counted using a Hemocytometer.

H. Co-culture of CD34$^+$ Hematopoietic Stem Cells with MSCs (Isolated and Mixed from Three Sources viz. Cord Tissue, Placental Tissue and Amniotic Tissue)

The mix of MSCs from three sources was harvested and seeded into a tissue culture-treated flask at a fixed density of 0.8 million cells, one day before the purification of CD34$^+$ hematopoietic stem cells. On the second day, the MSCs were observed under the microscope and checked for presence of cell adherence in a very low confluence. The MSC media was changed to CD34$^+$ cell expansion media containing IMDM (Gibco, USA) with 10 % serum (Gibco, USA), 35 ng/ml or 70 ng/ml or 100 ng/ml or 140 ng/ml (four sets) each of SCF (Peprotech, Israel), TPO (Peprotech, Israel), FLT3 (Peprotech, Israel) and 2 ng/ml bFGF (Invitrogen, USA). The purified CD34$^+$ hematopoietic stem cells were then seeded at a concentration of 5 million cells per flask and the cells were allowed to grow and expand over monolayer culture of MSC mix at 37^{0}C and 5% $CO_2$with humidity above 90%. The media was changed every third day and the cells were sub-cultured upon confluence, a maximum of up to two times in the total period of expansion of 14 days. On day 14, the suspended CD34$^+$ hematopoietic stem cells were collected in a 50 ml sterile tube and centrifuged at 1300 rpm for 5 minutes. The pellet was resuspended in a requisite volume of media and aliquots were obtained for cell count, flow cytometry and functional assay. The monolayer MSC mix in the flasks was harvested following routine internal protocol and the pellet was subjected to cell count, flow cytometry and functional assay. The 'MSC' mix pellet showed a small CD34$^+$ cells' contamination that was unavoidable.

I. Optimization of Growth Factor/Cytokine Concentration

The growth factors involved in expansion and maintenance of stem cells state of HSCs were optimized with respect to their final concentration in the expansion media. This was to understand the best possible growth factor concentration at which higher fold expansion can be achieved. The concentrations of growth factors were 35 ng/ml or 70 ng/ml or 100 ng/ml or 140 ng/ml (four sets) each of SCF (Peprotech, Israel), TPO (Peprotech, Israel), FLT3 (Peprotech, Israel) and bFGF in the HSC expansion media as described in point no. 2.8. Rest of the procedure remains the same.

J. Functional assay

500 expanded CD34$^+$ cells were taken per 35mm dish and mixed with 1.5 ml methylcellulose media (Stem Cell Technologies, USA). This suspension was then plated in duplicates and the plates were incubated at 37^{0}C and 5% CO_2 in a humidified environment. After 14 days of incubation, colonies were enumerated according to their type using a phase contrast microscope. The evaluation was performed by two individuals separately. They were proficiency checked.

Statistical Analysis

The data was analysed using student's unpaired 2 tailed t-test using online calculator provided by www.graphpad.com to determine statistical significance. Values are given as mean ± SD (standard deviation).

III. RESULTS

A. Characterization of MSCs Derived from Cord Tissue, Placenta and Amniotic Sac

The mix of MSCs from three sources viz. cord tissue, placental tissue and amniotic tissue revealed a typical 'fibroblast' like morphology under the microscope as shown in **Fig. 1(a).** Individual MSCs did not reveal major morphological

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

differences**.** Before reaching confluence MSCs appeared stellate, and as the culture progressed they turned fibroblastic in appearance. The mix of MSCs (n=7) expressed cell surface markers namely CD90, CD73 and CD105 and were negative for HLA-DR. The average viability of all MSCs (n=7) was above 98% by flow cytometry. [**Fig. 1(b**)]. Our observations are in line with the ISCT guidelines on MSC identification. Regardless of the source of MSCs, they were found to be positive for cell surface markers CD90, CD73 and CD105 and negative for HLA-DR [22].

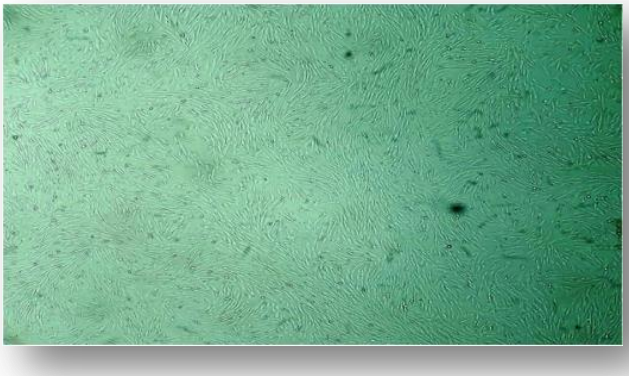

Figure 1(a): Mixed 'MSC' Culture showing 'fibroblastic' appearance. **[10X].**

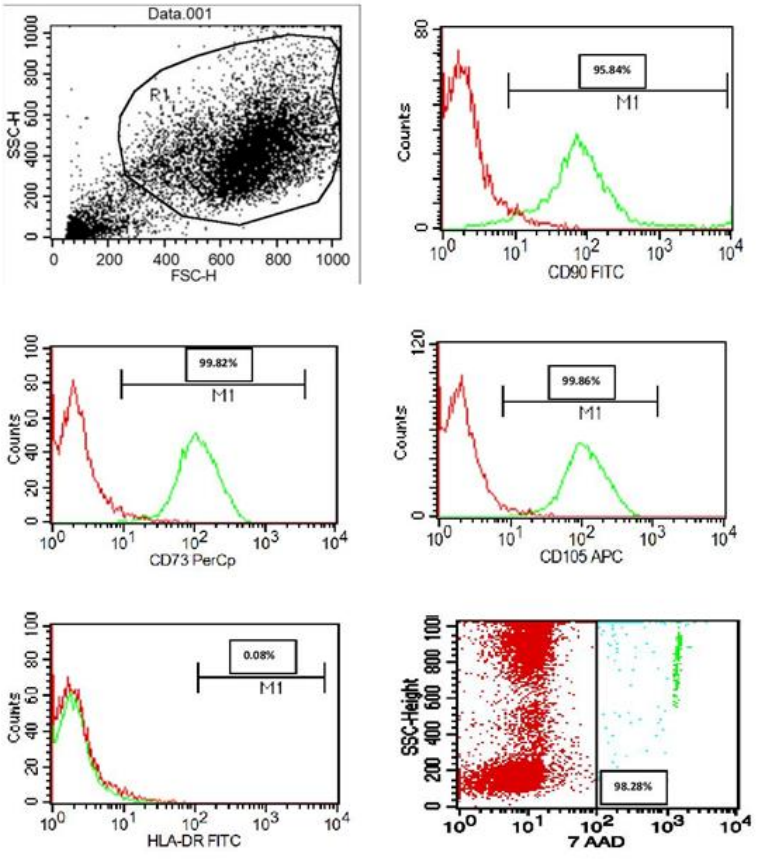

Figure 1(b): Representative (n=6) Flow cytometric plots of MSC mixture: CD90, CD73, CD105and HLA-DR expressed as 95.84%, 99.82%, 99.86% and 0.08% respectively. The viability was at 98.28%.

B. Enumeration of Umbilical Cord Blood CD34$^+$ HSCs

Umbilical cord blood HSCs were characterized and enumerated following ISHAGE protocol using flow cytometry as shown in **Fig. 2**.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

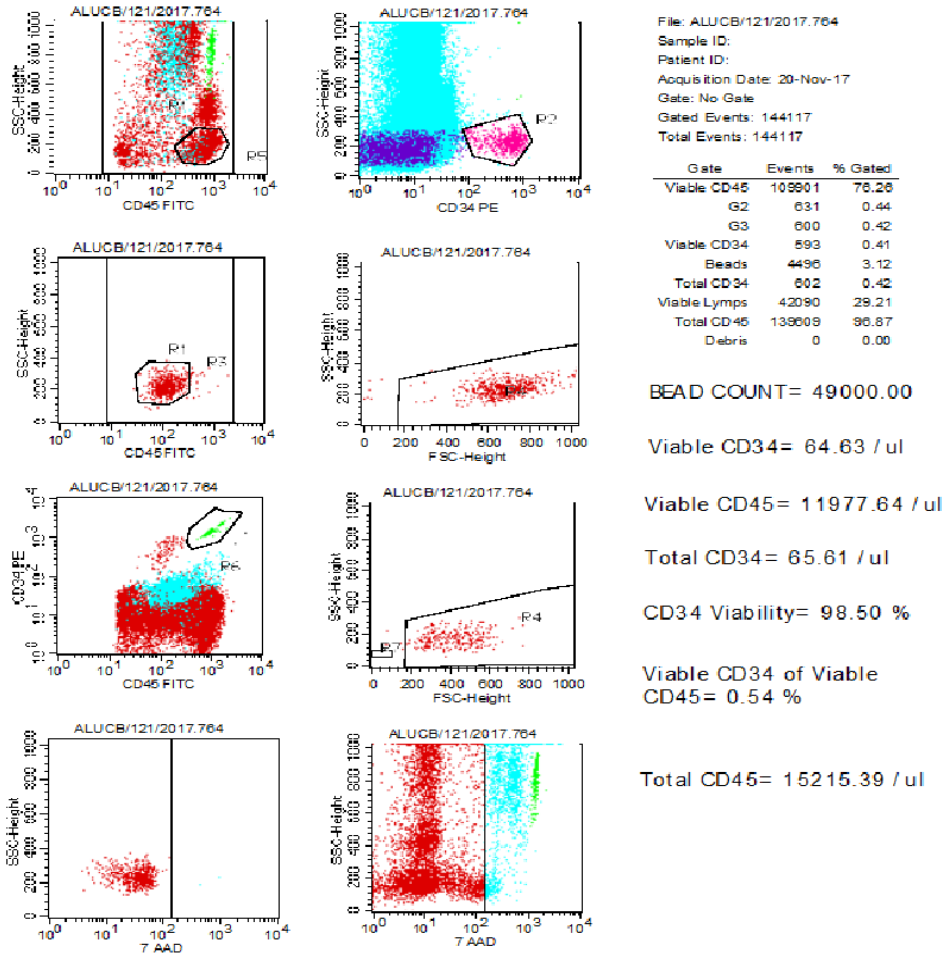

Figure 2: Representative (n=33) CD34$^+$ HSC from cord blood unit enumeration by ISHAGE Protocol

C. CD34$^+$ HSC Enumeration After Purification

The CD34$^+$ HSCs were typically round and shiny in the culture flask **(Fig. 3(a))**. **Fig. 3(b)** shows cells positive for CD45 (6.55 %) and CD34 (0.52%) immediately after purification but before expansion. The average viability was over 98%.

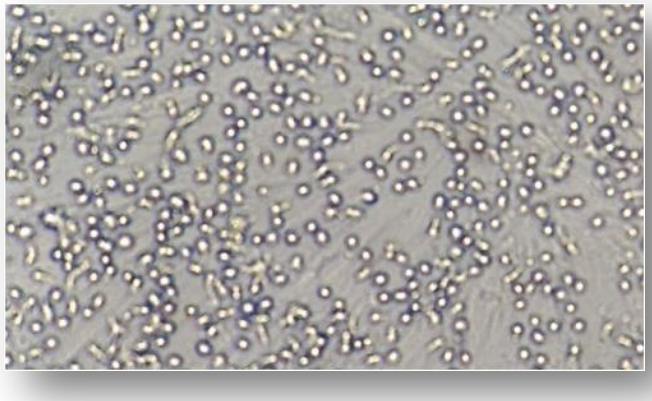

Figure 3(a): Morphology of CD34$^+$ HSCs Purified by Magnetic Labeling Technique From Umbilical Cord Blood Unit. [10X].HSCs seen as round, shiny cells floating in liquid

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

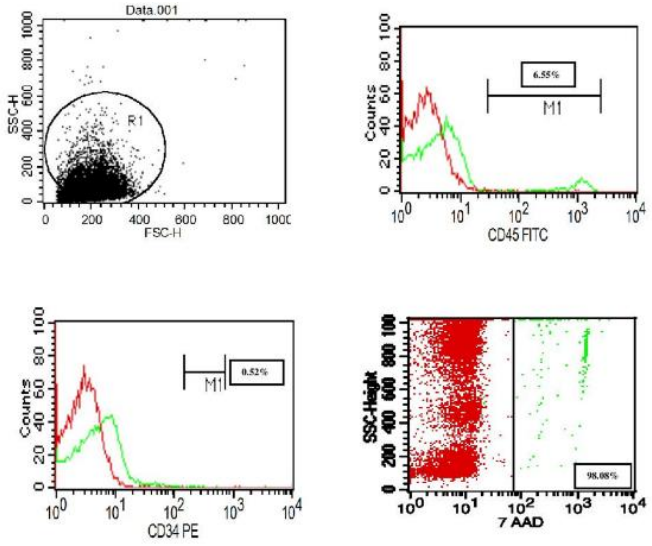

Figure 3(b): Representative (n=6) Flow Cytometric Immunophenotyping and Viability of Purified CD34$^+$ HSCs: The percent CD45 and CD34 were 6.55% and 0.52% respectively.

D. Morphology & Flow Cytometric Enumeration of Expanded CD34$^+$ HSCs

The expanded CD34$^+$ HSCs were characterized by microscopy. It revealed a typical round; shiny appearance of cells in bunches in the culture flask **(Fig. 4(a))**. The average (mean ± SD) fold expansion of CD34$^+$ HSCs was 29.60 ± 1.14 **(Fig. 6(a))**. The CD34$^+$ HSCs were positive for CD45 (99.24%) and CD34 (93.27%) markers as shown in **Fig. 4(b)**. The average viability was found to be above 98%.

Fig. 5 showing pictorial day-wise progress of CD34$^+$ HSC expansion over MSC feeder layer.

The role of initial CD34$^+$ HSC seeding density on HSC expansion is seen in **Fig. 6(b)**. The average (mean ± SD) expansion was at 376.20 ± 55.10 million from an average (mean ± SD) initial seeding numbers of 12.36 ± 1.63 million HSCs, which is statistically significant. ($p<0.0001$).

The relation between fold expansion and potency is shown in **Fig. 6(C)**. The average (mean ± SD) fold expansion of the five batches was 29.60 ± 1.14 and the average (mean ± SD) CFU (Colony Forming Units) were 23.99 ± 3.05 million on day 14. There is a linear correlation between the number of cells and its ability to form functional colonies, p value being 0.004.

Figure 4(a): Representative (n=6) Picture of CD34$^+$ HSC Morphology at Day 14. [10X].

Note: CD34$^+$ HSCs suspended in media. They exhibit confluence forming grape-like appearance. Observe the MSC-mix feeder layer in the background.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

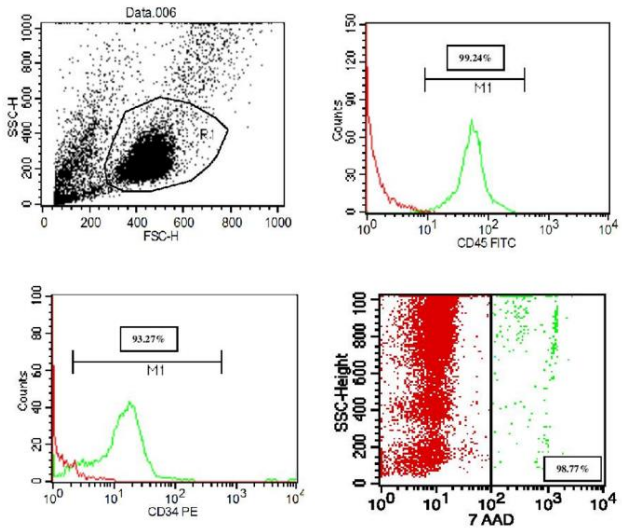

Figure 4(b): Representative (n=6) Flow cytometric Immunophenotyping and viability of expanded CD34$^+$ HSCs. The percent CD45 and CD34 were 99.24% and 93.27% respectively. The percent CD34 marker obtained was back-calculated with the total number of nucleated cells to arrive at the absolute numbers. The viability was 98.77%.

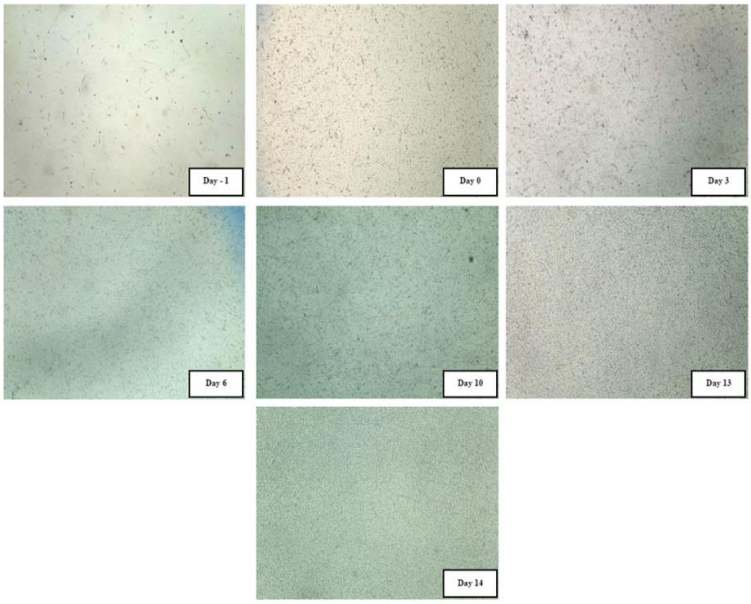

Figure 5) Representative Images of Day-wise Progress of CD34$^+$ HSCs in Culture. [10X]

E. Functional Characterization

Colonies of CFU-GM, CFU-GEMM and BFU-E were counted under the microscope **(Fig. 8)** and reported by a trained and proficient laboratory personnel twice and then averaged. The average total (mean ± SD) CFUs (Colony Forming Units) were 23.99 ± 3.05 million and we observed that the fold expansion affects CFU directly **(Fig. 6(c)).**

Table I) Colony Forming Unit (CFU) Assay for expansion batches having 100 ng/ml Growth Factor Concentration

Sr. No.	Batch No.	Fold Expansion	CFU (Million)
1	Batch 1	31	27.03
2	Batch 2	30	25.10
3	Batch 3	29	21.32
4	Batch 4	28	20.21
5	Batch 5	30	26.32

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

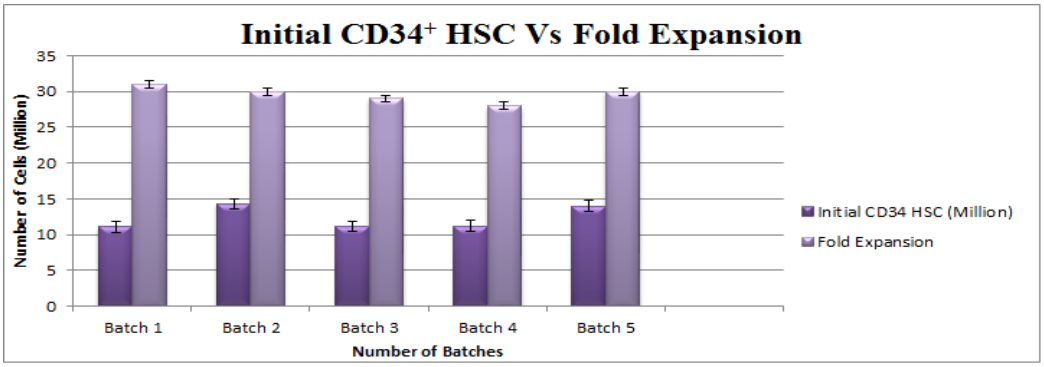

Figure 6(a): Inter-batch comparison of Fold Expansion of CD34$^+$ HSCs.

Initial seeding density of CD34$^+$ HSCs was compared with the fold expansion. The average (mean ± SD) fold expansion among five batches was 29.60 ± 2.75 and the average (mean ± SD) initial seeding density was 12.36 ± 1.63. Note that there is no linearity between cells seeded and fold expansion.

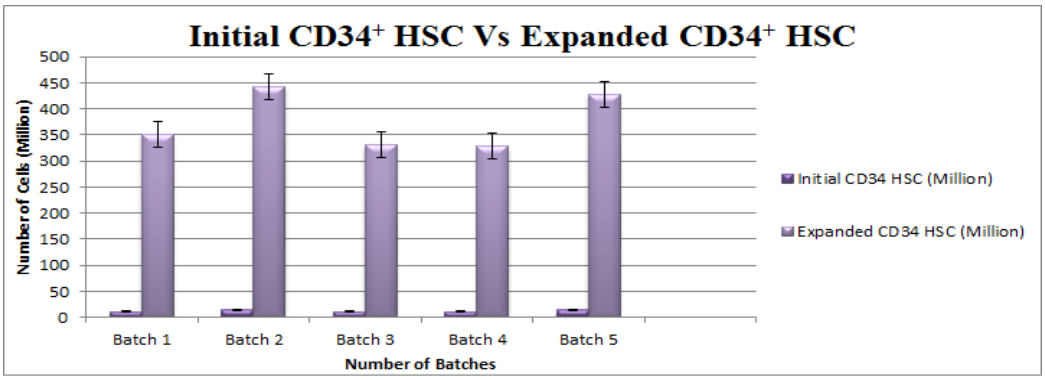

Figure 6(b): Consistency Data, Showing Dependable Expansion, From Batch to Batch.

Initial seeding density of CD34$^+$ HSCs was compared with the number of expanded HSCs after 14 days. The average (mean ± SD) expansion density was 376.20 ± 22.7 million from average (mean ± SD) initial seeding density of 12.36 ± 1.63 million HSCs. The increase in number of HSCs after expansion was highly statistically significant when compared with average seeding density of HSCs ($p<0.0001$).

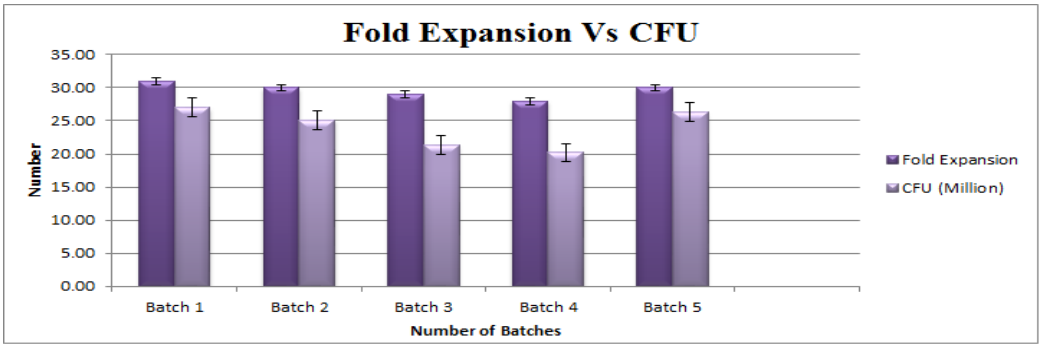

Figure 6(c): Relation between Cell Numbers and Functionality

Higher the expanded cell numbers, more the CFU formation. ($p=0.004$).

F. Optimization of Growth Factor/Cytokine Concentration

We used cytokines like Stem Cell Factor (SCF), Thrombopoietin (TPO) and FLT3 Ligand in four concentrations. These four concentrations were set 1 (35 ng/ml), set 2(70 ng/ml), set 3(100 ng/ml) and set 4 (140 ng/ml). The highest fold expansion among these set of cytokine concentrations was considered as an indicator of suitable cytokine concentration. The average fold expansion with set 1 was 14, set 2 was 20.2, set 3 was 29.6 and set 4 was 22.8 as shown in table II. The

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 6, Issue 4, pp: (1-14), Month: October - December 2018, Available at: www.researchpublish.com

fold expansion kept increasing with increasing cytokine concentration up to 100ng/ml, however, the fold expansion declined with cytokine concentration of 140 ng/ml (set 4).

Table II) Compilation of Parameters from Five Batches

Sr. No.	Batch No.	MSCs Seeded (Million)	Initial CD34 HSC (Million)	Expanded CD34 HSC (Million)	Fold Expansion	Cytokine Concentration (ng/ml)
1	Batch 1	0.6	15.71	185	11	35
			12.31	249	19	70
			11.1	351	31	100
			12.5	291	22	140
2	Batch 2	0.6	11.7	223	18	35
			12.42	281	22	70
			14.3	443	30	100
			13.6	346	24	140
3	Batch 3	0.6	12.69	172	13	35
			10	225	22	70
			11.2	331	29	100
			12.4	311	24	140
4	Batch 4	0.6	11.17	175	15	35
			14.9	299	19	70
			11.21	328	28	100
			13.2	298	22	140
5	Batch 5	0.6	15.05	211	13	35
			15	301	19	70
			14	428	30	100
			13.7	317	22	140

Growth Factor/cytokine concentration were varied as indicated. Note consistent fold expansion figures in media containing growth factor concentration 100 ng/ml.

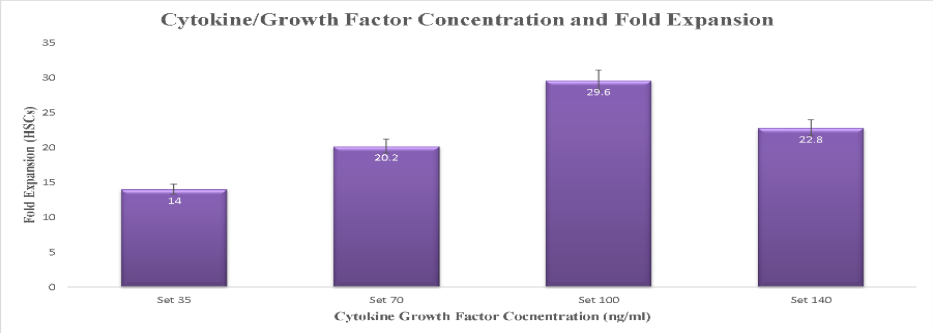

Figure 7) Fold Expansion Comparison among different Cytokine concentrations

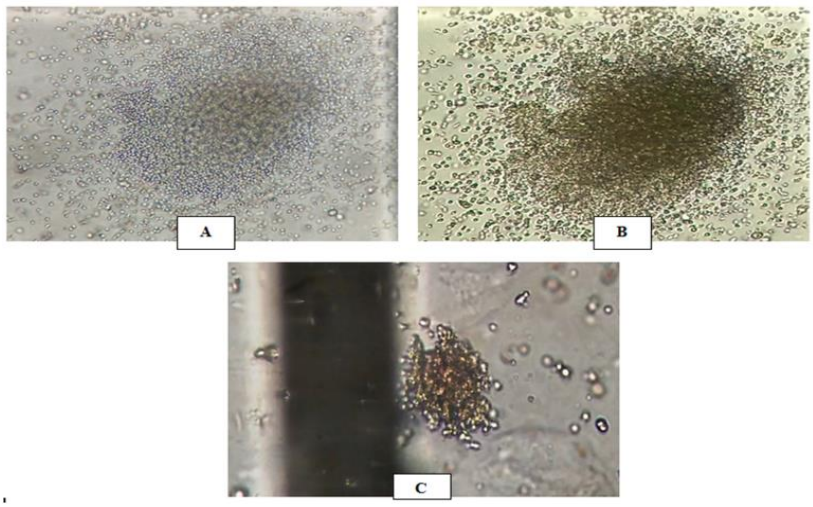

Figure 8) Representative Pictures of Colony Forming Units (CFU) on day 14 after plating of expanded $CD34^+$ HSCs: (A) CFU-GM Colony (B) CFU-GEMM Colony (C) BFU-E Colony. [10X].

IV. DISCUSSION

The outcome of $CD34^+$ HSC transplantation is often adversely affected due to low cell numbers in the cord blood graft [3]. For this reason, efforts are being directed towards validating novel and cost effective expansion protocols to meet the increasing demand of hematopoietic stem cells [5][6][7][8][9]. The HSCs, apart from reconstituting haematopoiesis play a significant role in the regenerative medicine arena. Kurtzberg and team from Duke University released findings from a double-blind, placebo-controlled phase II clinical trial and established Safety and efficacy of umbilical cord blood stem cells for the treatment of Cerebral Palsy [23]. The same group is testing the feasibility and safety of umbilical cord blood stem cells in patients with Autism Spectrum Disorder (ASD) [24]. There are several studies that are currently ongoing, on the use of HSCs for various other degenerative disorders.

In this study, the umbilical cord blood units were collected, processed and stored within 24 hours of collection as per the cord blood banking protocols. These cord blood units were used for HSC expansion within one year of cryopreservation. Prior to use, they were thawed as per our internal processes. Multiple cord blood units were retrieved and pooled for expansion purposes and the starting number of cells for expansion was defined. HLA matching was not considered as they were meant to be for vasculogenesis or angiogenesis. It is postulated that they work through paracrine signalling or by increasing the local cues [15]. The evidence of this non HLA matched transfusion can be traced back to as early as the 1930s, when there was no HLA matching technology available [15]. In those studies, the blood group matched and HLA mismatched cord blood units were transfused to a non-preconditioned patient with neonatal anaemia [25].The purpose then probably was for improving oxygenation. But for hematopoietic reconstitution, only the best HLA matched unit/s shall be expanded to the desirable levels. The challenge there is to get the right match from a repository and expand it meaningfully, for a hematopoietic reconstitution. In Africa, 128 paediatric patients with severe anaemia were transplanted with ABO-matched but HLA-mismatched cord blood with no reports of any graft versus host disease [26]. While pooling samples for expansion, invariably, all samples have different HLA types. The cost for expanding a single cord blood unit versus pooled units was much higher as it was the starting count that decided the final numbers. So, it is wise to use expensive consumables judiciously on 'pooled' units and expect proportionately higher number of cells. We have assumed that mixing dissimilar HLAs in culture has not affected the expansion dynamics. In our experiment, we tried to stay focused on making it cost effective by optimizing all parameters, mainly the cytokine quantities. We used the same media as reported by Tipnis et al [3] but with different concentrations of cytokines. The purpose of using different cytokine concentrations was to find out the best concentration at which higher fold expansion could be possible. We observed an average 30 fold expansion with 100 ng/ml of cytokine concentration being highest among all other concentrations of cytokines tried in this study. This was a valuable observation. The feeder layer in our study is a mix of naive cells as compared to studies that chose cells from a single source like bone marrow and cord tissue.

Instead of the traditional Trypsin used for cell harvesting, we used a recombinant Trypsin (Trypsol-AOF®, Biogenomics, India) which was gentle on the cells and is animal protein free. The Trypsol-AOF® was very useful, and can definitely support cell survival. This 'animal free' reagent would be a regulatory requirement as cells move into the clinical trial phase. We conducted validation batches on a pilot scale using a fixed initial seeding density of 10-15 Million cells. We have not tried increasing or decreasing this density to check for optimization. This fixed density may or may not have led to any feedback inhibition, though we have not been able to demonstrate that.

It is known that several factors other than growth factors and cytokines affect $CD34^+$ HSC expansion. Needless to say, this includes besides other factors, the proficiency and training of the personnel performing the experiment, the quality of various cord blood units retrieved and the nature of MSCs.

The cells constituting the feeder layer affects HSC expansion to a great extent. So far, MSCs derived from various sources have been checked for their suitability as a feeder layer [3]. In this experiment, we tried a mix of MSCs derived from three perinatal tissues i.e. umbilical cord tissue; placenta and amniotic sac as shown in figure 1(a). The phenotype of MSC mix is as shown in figure 1(b). It complied with the ISCT guidelines for identification of MSCs [27]. The nature and source of feeder layer reportedly has a direct impact on prevention of apoptosis of expanded HSCs [28]. This perhaps is believed to be due to various soluble factors and signalling cues secreted by MSCs [29]. Wagner *et al* reported that there is genetic variability among MSCs derived from different sources [30] and is reflected by the difference in the levels and quality of growth factors secreted by them [31]. Although our objective was not to study the effect of feeder layer on apoptosis of HSCs, we got a viability of >98% in all the batches as shown in figure 4(b). This is an indirect proof

suggesting lesser cell losses in the process. According to Klingemann et al, the MSCs from perinatal sources (cord tissue, placenta and amniotic sac) are ontogenetically more primitive as compared to those from adult sources. This is accompanied by considerable functional variations [32][33] and generation of more anti-apoptotic signals. This was favourable and desirable.

Next, we checked the role of MSC numbers as a feeder layer. More the MSCs faster is the growth rate, and chances of inhibition of HSC multiplication. And, too few MSCs, slow down the expansion. Hence there is very delicate balance between growth kinetics of MSCs and HSCs. We observed that with a small inoculum of 0.6 million MSCs in a T175 flask, 10-12 million HSCs can be seeded for a good expansion (Refer Table 1). We achieved consistent results in all batches processed.

The average (mean ± SD) fold expansion of the five batches was 29.60 ± 1.14 (Figure 6(a)) which is nearly same as reported in previous studies using MSCs derived from umbilical cord tissue. This outcome is also consistent with the study reported by McNiece et al using bone marrow-derived MSCs [34].

The morphology of HSCs prior to and after expansion remained unaffected as shown in figure 3(a) and 4(a). We observed a linear and exponential relationship between fold expansion and CFU outcomes using a cost effective process. Cell numbers directly affected the potency (CFU) of the expanded HSCs ($p=0.004$). The duration of cryopreservation prior to expansion did not affect the expansion outcome in any way.

V. CONCLUSION

We showed reproducible results with regards *ex-vivo* expansion of $CD34^+$ HSCs using a cost effective protocol with the use of mix of MSCs derived from different perinatal sources. This study is the first of its kind where unrelated Mesenchymal Stem Cells (MSCs) derived from three different perinatal sources (viz. cord tissue, placenta and amniotic sac) were used as a feeder layer for $CD34^+$ HSC *ex-vivo* expansion.

This cost effective $CD34^+$ HSCs expansion protocol is expected to be useful in addressing various clinical needs in the future. These cells are promising to be the next candidate cell type that will find place in the treatment of degenerative disorders.

CONFLICT OF INTEREST

The authors do not have any conflict of interest.

CONTRIBUTIONS

1) **Sushilkumar Ramdasi:** Extensive literature review. Validated entire protocol of $CD34^+$ HSC isolation, purification, characterization and expansion. Conducted all batches. Compilation of results. Preparation and subsequent revisions of the manuscript.

2) **Chandra Viswanathan:** Strategy, planning, and Proof of concept for $CD34^+$ HSC expansion using various approaches. Provided extensive mentoring, guidance and motivation. Guided in the preparation of the manuscript and in the compilation of the results. Extensively reviewed & proof-read the manuscript.

3) **Rohit Kulkarni:** Provided financial resources for the project work & Manuscript reviewed.

4) **Abhijit Bopardikar:** Provided necessary guidance and resources for the project work. Manuscript review.

5) **Raghwendra Kumar:** Provided Umbilical Cord Blood units required for expansion and provided MSCs required for feeder layer.

6) **Pradeep Somalapur:** Provided characterization of Umbilical Cord Blood units required for expansion and provided MSCs required for feeder layer.

7) **Poonam Pandey:** Characterization and assistance in cell culture for HSC expansion. Monitoring of quality aspects of research.

REFERENCES

[1] Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med 1997;337:373-381.

[2] Wagner JE, Rosenthal J, Sweetman R, et al. Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. Blood 1996;88:795-802.

[3] Tipnis S, Viswanathan C. Umbilical cord matrix derived mesenchymal stem cells can change the cord blood transplant scenario. Int J Stem Cells. 2010 May;3(2):103-18.

[4] Viswanathan C, Kulkarni R, Bopardikar A, Ramdasi S. Significance of CD34 Negative Hematopoietic Stem Cells and CD34 Positive Mesenchymal Stem Cells - A Valuable Dimension to the Current Understanding. Curr Stem Cell Res Ther. 2017;12(6):476-483.

[5] Tepper OM, Capla JM, Galiano RD et al. Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. Blood 2005;105:1068-77.

[6] Jang YY, Collector MI, Baylin SB, et al. Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 2004;6:532–539.

[7] Zhang S, Wang D, Estrov Z, et al. Both cell fusion and transdifferentiation account for the transformation of human peripheral blood CD34-positive cells into cardiomyocytes in vivo. Circulation 2004;110:3803-3807.

[8] Mao Q, Chu S, Ghanta S, et al. Ex vivo expanded human cord blood-derived hematopoietic progenitor cells induce lung growth and alveolarization in injured newborn lungs. Respir Res 2013; 14:37.

[9] Taguchi A, Soma T, Tanaka H, et al. Administration of $CD34^+$ cells after stroke enhances neurogenesis via angiogenesisin a mouse model. Journal of Clinical Investigation. 2004;114(3):330-338.

[10] Benjamin Dekel, A Elias Shezen, A Smadar Even-Tov-Friedman, et al. Transplantation of Human Hematopoietic Stem Cells into Ischemic and Growing Kidneys Suggests a Role in Vasculogenesis but not Tubulogenesis. Stem Cells 2006;24:1185-1193.

[11] Dzau VJ, Gnecchi M, Pachori AS et al. Therapeutic potential of endothelial progenitor cells in cardiovascular diseases. Hypertension 2005;46:7-18.

[12] JL Goldberg and MJ Laughlin. UC blood hematopoietic stem cells and therapeutic angiogenesis. Cytotherapy 2007;9(1):4-13.

[13] Dunac A, et al. Neurological and functional recovery in human stroke are associated with peripheral blood CD34+ cell mobilization. J Neurol. 2007;254(3):327–332.

[14] Riordan NH, Chan K, Marleau AM, Ichim TE. Cord blood in regenerative medicine: do we need immune suppression? Journal of Translational Medicine. 2007;5:8.

[15] Zeman K, Fornalczyk-Wachowska E, Pokoca L, et al. The composition of main lymphocyte's subsets and NK cells in peripheral blood of the Polish population. Cent Eur J Immunol 1996;21:107-113.

[16] Jedrasiak U, Radomska I, Kepinska M. Leukocytic parameters in the umbilical blood of full-term neonates-on the basis of authors experience. Nowa Ped 1999;3:3–6.

[17] Knudtzon S. In vitro growth of granulocytic colonies from circulating cells in human cord blood. Blood 1974;43:357-61.

[18] Nakahata T, Ogawa M. Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. J Clin Invest 1982;70:1324-8.

[19] Broxmeyer HE, Douglas GW, Hangoc G et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Natl Acad Sci USA 1989;86:3828-32.

[20] Shetty P, Cooper K, Viswanathan C. Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. Asian J Transfus Sci 2010;4:14-24.

[21] D. Robert Sutherland, Lori Anderson, Michael Keeney, et al. The ISHAGE Guidelines for CD34+ Cell Determination by Flow Cytometry. Journal of Hematotherapy 2009,5(3):213-226.

[22] HEO JS, CHOI Y, KIM H-S, KIM HO. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. International Journal of Molecular Medicine 2016;37(1):115-125.

[23] Clinical trial: Joanne Kurtzberg, MD. A Randomized Study of Autologous Umbilical Cord Blood Reinfusion in Children with Cerebral Palsy. ClinicalTrials.gov Identifier: NCT01147653.

[24] Geraldine Dawson, Jessica M. Sun, Katherine S Davlantis, et al. Autologous Cord Blood Infusions Are Safe and Feasible in Young Children with Autism Spectrum Disorder: Results of a Single-Center Phase I Open-Label Trial. Stem Cells Translational Medicine 2017;6:1332-1339.

[25] Halbrecht J. Fresh and stored placental blood. Lancet 1939;2:1263-65.

[26] Hassall O, Bedu-Addo G, Adarkwa M, et al. Umbilical-cord blood for transfusion in children with severe anaemia in under-resourced countries. Lancet 2003 Feb 22;361(9358):678-9.

[27] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315-7.

[28] Mehrasa R, Vaziri H, Oodi A, et al. Mesenchymal Stem Cells as a Feeder Layer Can Prevent Apoptosis of Expanded Hematopoietic Stem Cells Derived from Cord Blood. International Journal of Molecular and Cellular Medicine 2014;3(1):1-10.

[29] Tung SS, Parmar S, Robinson SN, et al. Ex vivo expansion of umbilical cord blood for transplantation. J Best Pract Res Clin Haematol 2010 Jun;23(2):245-57.

[30] Wagner, Wolfgang et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 2005;33(11):1402-1416.

[31] Friedman, Robb et al. Umbilical Cord Mesenchymal Stem Cells: Adjuvants for Human Cell Transplantation. Biol Blood Marrow Transplant 2007;13(12):1477-1486.

[32] Klingemann H, Matzilevich D, Marchand J. Mesenchymal Stem Cells – Sources and Clinical Applications. Transfusion Medicine and Hemotherapy 2008;35(4):272-277.

[33] Bastiaan J.H. Jansen, Christian Gilissen, et al. Functional Differences Between Mesenchymal Stem Cell Populations Are Reflected by Their Transcriptome. Stem Cells and Development 2010;19(4):481-490.

[34] McNiece I, Harrington J, Turney J, et al. Ex vivo expansion of cord blood mononuclear cells on mesenchymal stem cells. Cytotherapy 2004;6:311-317.