RESEARCH ARTICLE

Genetic reprogramming of cord blood derived endothelial colony forming cells towards human induced pluripotent stem cells using episomal plasmids

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Abstract

Objective: This study aimed to isolate human umbilical cord blood derived endothelial colony forming cells (ECFCs) followed by their integration free reprogramming towards induced pluripotent stem cells (iPSCs) and molecular characterization of both cell types using multicolour flowcytometery and immunofluorescence respectively.

Methods: The cord blood was collected from 37-39 weeks of gestational ages after C-section ex-utero from Dow University Hospital. The ECFCs isolated after ficoll based separation of cord blood mononuclear cells (CBMNCs) which on emergence characterized through flow cytometry and reprogrammed towards induced pluripotent stem cells (iPSCs) using episomal vectors, the iPSCs were characterized using immunofluorescence. The study was conducted at Stem Cells and Regenerative lab, Dow Research Institute of Biotechnology and Biomedical Sciences, Dow University of health sciences OJHA campus. The study time duration was about one year (October 2017-October 2018); study design was in vitro experimental. The sample size of the study was n=3.

Results: The isolated ECFCs were evaluated using flowcytometery which showed positive expression for CD31, CD34, CD146 cell surface markers and negative for CD90. The successful reprogramming of ECFCs towards iPSCs was confirmed by immunofluorescence using OCT-4 which is considered to be a master regulator of pluripotency.

Conclusion: To the best of our knowledge this study was the first attempt to integration free reprogramming of cord blood derived endothelial colony forming cells towards induced pluripotent stem using episomal plasmids. Cells that have been isolated from cord blood and those that have been reprogrammed both have potential therapeutic applications in regenerative medicine.

Keywords: Regenerative medicine, Human umbilical cord blood, Endothelial colony forming cells, Episomal reprogramming, Induced pluripotent stem cells. (JPMA 71: 1081; 2021)

DOI: https://doi.org/10.47391/JPMA.465

Introduction

Stem cells deciphering is the way of our understanding in ailments of different conditions as well as in conquering to reveal the hidden secrets underlying mechanisms of previously known and unknown diseases. In this study, we use cord blood derived endothelial colony forming cells or simply ECFCs, cord blood being young and proliferative source, is a potential candidate for regenerative use. The ECFCs have vasculogenic as well as angiogenic property and enables them to be the valuable candidate for regenerative use in a variety of ways and this population arises from progenitors in the cord blood or peripheral blood and after isolation, they have all features of endothelial lineage.[1] There are three cascades which define vasculature formation, one is through angiogenesis which is from the pre-existing vessel, other is vasculogenesis that occurs from localized angioblasts or endothelial precursor cells and in case of an event, collateral arteriogenesis take place by the migration of endothelial cells towards the injured site.[2-4]

This study enlightens the approach towards the clinical use of human umbilical cord blood as it is a valuable source for regenerative therapy all over the world but it is discarded as a medical waste in our country. This will be the first study published from Pakistan on cellular reprogramming of an adult cell type (cord blood derived ECFCs) towards induced pluripotent stem cells. To the best of our knowledge Episomal plasmids have not been used for reprograming of ECFCs anywhere before.

ECFCs; Role and Importance

ECFCs play an important role in vascular repair, these are the type of endothelial progenitor cells or EPCs which appears in colony and have high proliferative capacity when derived from cord blood which is an enriched source for these cells, the number of ECFCs are up to 15 fold high in cord blood in comparison with peripheral

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blood as well they appear earlier and larger from cord blood and these ECFCs contain grading according to population doubling and capacity to produce clusters or colonies on replating[5]. High proliferative potential ECFCs or HPP-ECFCs and low proliferative potential ECFCs or LPP-ECFC can form secondary and tertiary colonies upon replating. HPP-ECFCs can give rise to secondary HPP-ECFC but LPP-ECFC are unable to form secondary LPP-ECFCs but they can form endothelial cell clusters consisting of less than 50 cells. These cells are unable to form clusters or colonies and yield mature endothelial cells,[5] ECFCs were first described by Asahara,[6] there is no such novel marker which could identify ECFC but a recent report suggests that CD133 in ECFCs may expresses CD133 intracellularly which could contribute to vasculogenesis.[7] These cells have the potential for screening toxicological and anti-tumour effects in-vitro using ECFCs cell line from human UCB, as it leads to tube formation on matrigel making it competitive enough for drug assay. Umbilical cord blood from term human placenta contains valuable cell sources which offer stem cells for therapeutics. Cord blood contains high colonogenic and proliferative endothelial progenitor cells or EPCs that forms cobblestone colony on appearance termed as endothelial colony forming cells or simply ECFCs as first described in detail by Yoder and Ingram.[5] In preclinical studies, ECFCs have proven effective for diabetic wound healing,[8] ischaemic retinopathies[9] which could be enhanced by Cibinetide as shown recently,[10] they also prove to be effective in encephalopathy[11] and EPCs were used in patients with acute ischaemic stroke and follow up for 4 years providing an evidence of their effectiveness.[12] The iPS after its discovery overcomes the hurdle for use of human ESC as there is a strong relationship in signature of the gene expression among iPS and ESC.[13]

Reprogramming and iPS Cells

Cord blood derived cells being young, proliferative and resemblance to the molecular signature with those of ESC cells are a valuable source for candidate of reprogramming towards iPS cells. iPS cells have paved their way after their discovery[14] and soon translated to human iPSCs the next year[15] and then continue to progress at improvement and providing the foundation for novel cellular treatments as well as drug innovations towards bed side.[16,17] Reprogramming offers the opportunity to explore the avenue of hidden findings in the oceans of diverse medical conditions and iPSCs have great potential to discover options for novel therapeutics.

The aspect of using stem cells for regeneration implies the use of cell or tissue based products for cellular based treatment as well as compounds screening of pharmacological and its related chemicals with perspective for treating inherited disorders.[18] The concept of drug discovery and personalized medicine using regenerative products is now increasing.

Materials and Methods

Consent approval

Informed consent was taken from the mother in the format as approved by the IRB (Ref: IRB-777/DUHS/Approval/2016/294) and BASR committee (Ref: DUHS/BASR/2016/-17, BASR Meeting No. 50) of Dow University of Health Sciences. Study was conducted for a period of around twelve months (October 2017-October 2018).

Umbilical Cord Blood Collection

Samples were collected as according to the guidelines of National Bioethics Committee for Stem Cell research in Pakistan[19] and as per Declaration of Helsinki after informed consent. About 60-120 ml of cord blood was collected after cleaning the collecting site with 70% alcohol swab using JMS Blood bag 250ml with 17G needle (Ref no. 811-2536, Reg no. 011442, JMS, Singapore Pvt. Ltd.) containing 35ml CPDA-1 as anticoagulant. After the placenta was delivered, venepuncture was performed for collecting cord blood. The blood bag was placed in a dependant position and was continously shaken for proper mixing of the cord blood with CPDA-1(anticoagulant). A second puncture site was then selected carefully above the first point. After cord blood collection the blood bag tubing was secured with 2-3 knots and transferred to the lab. It is recommended to process the sample within 2-4 hours and maximum time limit for sampling process is about 12-15 hours. Our collection procedure was according to the protocols as previously mentioned[20] with little changes.

CBMNCs Isolation

Samples were processed within 2-4 hours after collection. The sample was collected after making PBS mix with a ratio of (1:1) then placed on ficol layer (Ficoll-Paque 1077, Cat no. 17-1440-03, GE Healthcare, Sweden). The sample was centrifuged at 800xg without brakes for 25-30 minutes, the buffy coat was aspirated with the precaution to keep out RBCs to prevent RBCs abundance. After ficol centrifugation, buffy coat was taken and mixed properly with 1X PBS and spinned at 800xg with high brakes and acceleration setting for 5 minutes as washing step. The washing was done 2-3 times as needed. All steps were followed as described by Yoder and Ingram[21] with some modifications.

Human ECFCs culture

ECFCs were cultured into c EGM-2 single quotes (Cat no. CC-4176 containing EGM-2 Bullet kit Cat no. CC3163, Clonetics, Lonza) with 2% FBS which was later increased up to 10-15%, the media was changed the next day or alternate day or as required according to the culture conditions with or without wash with 1XPBS 1.5-2.5ml for 6well, 1-1.5ml for 12 well and 0.5-1ml for 24 well and after getting colony, ECFCs were grown, split and passaged through colony picking, trypsinization using trypsin/EDTA (Cat no. R001-100, life tech) or trypLE (Cat no. 12604013, thermo scientific) Express or via cell scrapper on fibronectin (50ug/ml) coated 24 well plate.

Multi-colour Flowcytometery of ECFCs

Flow analysis of ECFCs was done on BD FACS Celesta using FACS DIVA software version 8.0, voltage of FSC sets on 350 and SSC on 250 at medium run at 10,000 events and flow rate adjusted to 240 events/sec. Tubes were mark stained as positive and unstained as negative control, mouse anti-human antibodies were used as CD 146 (Cat no. 563186) FITC labelled CD31 (Cat no. 560984) APC labelled CD 34 (Cat no. 560940) CD 90 (Cat no. 562686) and anti-mouse Igκ BD Comp Bead Plus for compensation (Cat no. 560497) all purchased from BD Pharmagen, the flow was carried out as mentioned[22] with some necessary optimization.

Transfection

The expanded ECFCs were transfected by using episomal reprogramming kit (Cat no. A15960, life technologies) that contains seven defined factors including Oct4, L-Myc, Sox2, Klf4, Lin28, p53 and SV40LT with the aid of neon nucleofection system at 1350V, 30ms pulse width and 1 pulse number by using 10ul neon tip for vectors induction. After completion, immediately transfer the transfected cells into the c-EGM-2 medium to a Vitronectin-N coated at concentration of 5µg/ml (Cat no. A14700, life tech) on a 12 well plate. On day 1 and 2 post-transfection, 0.5 ml of N2B27 media; containing N2 supplement (100X) Cat no. 17502048, B27 serum free supplement (50X) Cat no. 17504044, DMEM/F12 with HEPES Cat no. 11330032, MEM NEA solution (10MM) Cat no. 11140050, β-Mercaptoethanol (1000X) Cat no. 21985023, Glutamax-1 supplement (100X) Cat no. 35050061, Basic FGF Cat no. PHG0264, Ultra-pure 0.5 M EDTA, pH 8 Cat no. 15575020 all from Life technologies was added. The old media was not aspirated. On day 3-6, 0.5 ml media was aspirated and 0.5 ml N2B27 media was added. On day 7, whole media was removed and shifted to 1ml N2B27 media while on day 8 media was changed from N2B27 to 1ml Essential 8 media (Cat no. A1517001 containing Essential 8 supplements (50X) Cat no. A1517101, life tech) which contains 8 essential components necessary for pluripotency. The method was as per protocol described by[23-26] with some optomizations.

Colony picking iPSCs

The reprogrammed cells after achieving iPSCs morphology were picked under DMi1 inverted phase contrast microsocpe and grown into VTN-N coated (5µg/ml) 24 well. These picked cells were subjected to immunostaining on the next day.

Immunofluorescence

The reprogrammed iPSCs were fixed at day 11 post transfection using acetone and methanol (1:1) and blocked into 2% BSA then stained with primary antibodies OCT4 as positive and SSEA-1 as negative from ESC maker kit (Cat no. SCR002, Chemicon USA) into blocking solution in a ratio of 1:50 then blocked with FITC labelled secondary antibody antihuman IgG goat (Cat No. AF102-0115) in a proportion of 1:200 and for nucleus staining, 10ul DAPI was used. Images were acquired and taken using Dmi8 inverted fluorescent microscope (Leica Microsystem, Germany), first into bright field at 20X on bright field filter then on FITC for FITC labelled antihuman IgG goat and DAPI for nucleus visualization and to acquire the images using LAS software version 4.8 (Leica Microsystems, Germany). The immunofluorescence was done as described earlier[27] with some necessary changes.

Results

Primary Culture

Culturing of ECFCs was carried out as stated in[1] with some modifications and cobblestone colony Figure-1a (ii & iii) appears from 5-11 days, after passaging and expansion, flow analysis for immunophenotyping of ECFCs was done using positive and negative markers. The isolation after cord blood collection was processed from the collected blood as shown in Figure-3 (a), total number of viable cells in terms of millions per ml as displayed in Figure-3 (b) and with varying levels of percentage viability as shown in Figure-3 (c).

Flow Cytometry

Since there is no single marker that can identify ECFCs bonafide population, set of markers were used to profile ECFCs using flow cytometry, CD 146 or MCAM (melanoma cell adhesion molecule) is used to identify endothelial cells as an important marker, gating was done using this marker and might contains blood cells as also stated earlier.[22] CD31 or PECAM (Platelet endothelial cell adhesion molecule) another marker used in combination with other positive markers to assess ECFCs was showing

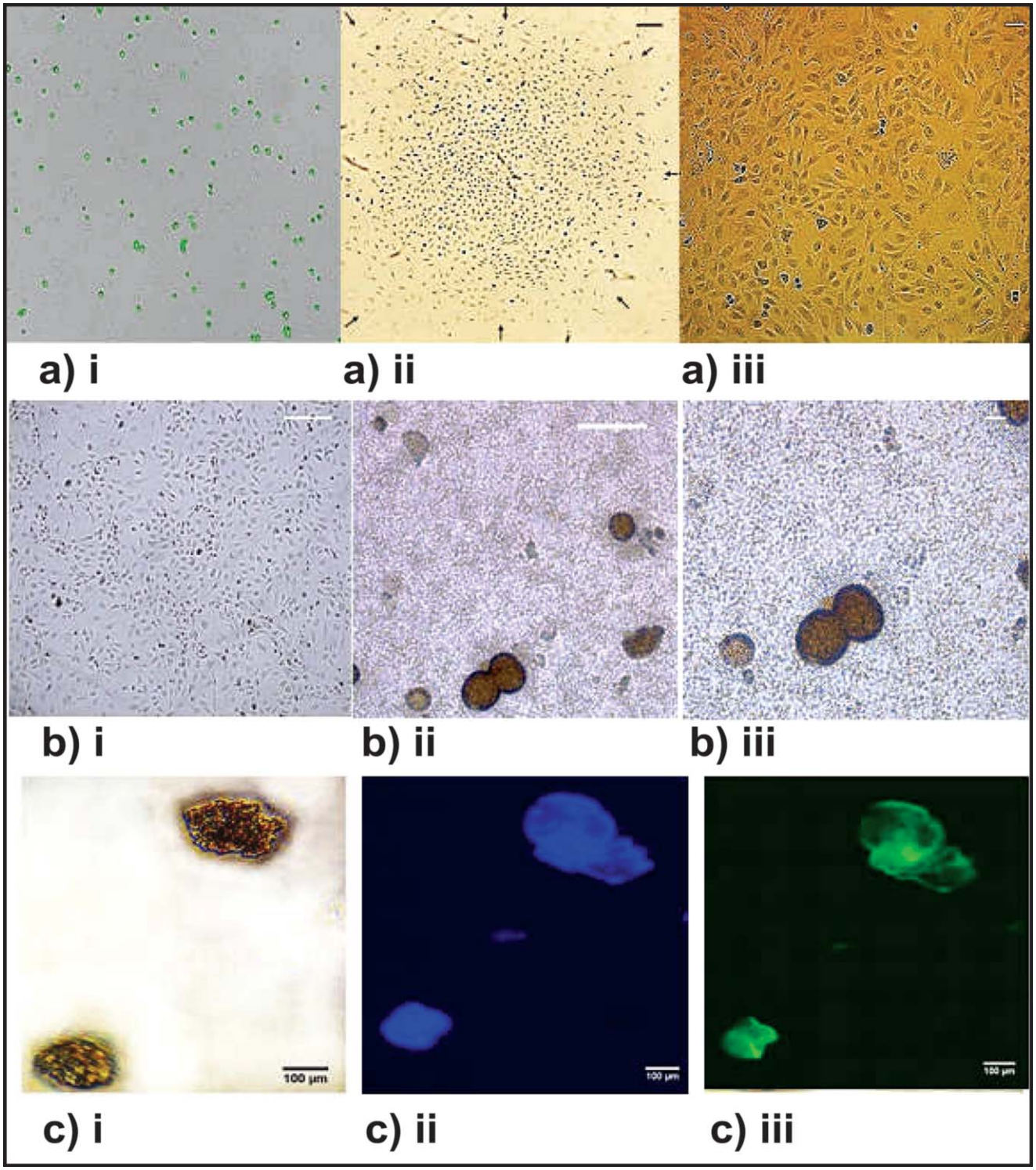

Figure-1. a) Viable cell analysis and Phase contrast microscopy of ECFCs i) Viable cells after isolation using vi cell counter (Beckman Coulter) ii) ECFC colony at 4x, arrow shows the distinct boundary of ECFC colony iii) ECFCs at 10X magnifications.

b) Transfected cells and iPSCs i) ECFCs used for transfection at 10X ii) Emerged iPSCs after transfection at 10X iii) iPSCs at 20X, Scale bar = 100µm. All images acquired on phase contrast microscope Leica Dmi1 using software LAS v 4.6.1 (Leica Microsystems, Switzerland).

c) Reprogrammed cells after Immunostainning i) Bright field image of iPSCs at 20 X ii) Immunofluorescence of iPSCs DAPI for nucleus staining at 20X iii) FITC labelled OCT4 at 20X, all images were taken using Leica Dmi8 on software LAS v 4.8 (Leica Microsystems, Switzerland). Image scale = 100µm.

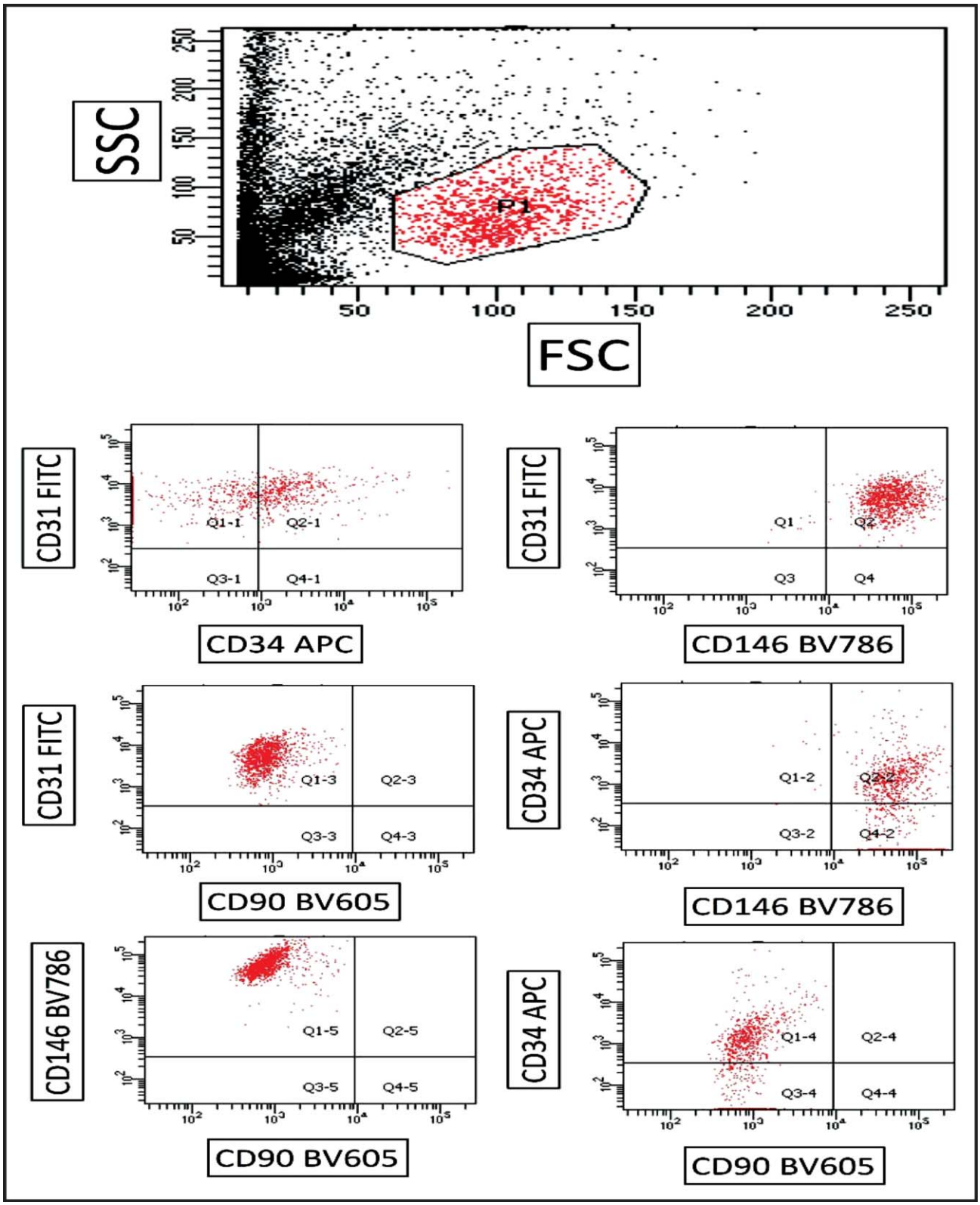

Figure-2: Flow data showing gated population SSC vs FSC, CD31 vs CD34, CD31 vs CD146, CD31 vs CD90, CD34 vs CD146, CD146 vs CD90 and CD34 vs CD90, the set of positive markers include CD31, CD146 and CD34 while CD90 as negative marker, the cell population evaluated using dot plot, all acquire in FACS Diva software version 8.0.
CD stands for cluster of differentiation; SSC stands for side scatter, FSC stands for forward scatter.

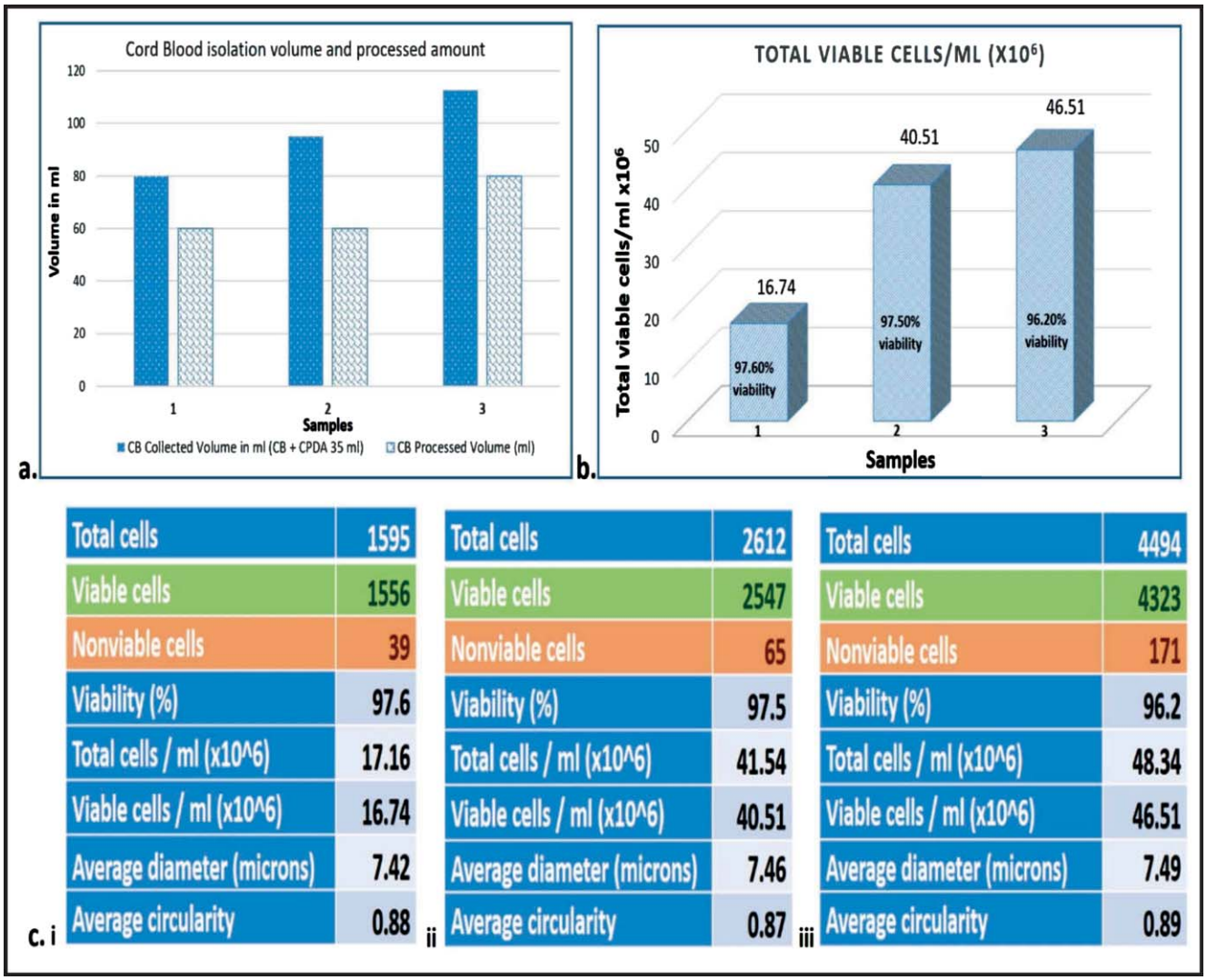

Figure-3: Charts and Vi cell data a) Showing cord blood collected volume vs the processed cord blood volume (n=3) b) Cells viability in percentage (%) and the total viable cells per ml (1x106) from cord blood processed volume (n=3), using Vi-Cell analyzer XR through Beckman Coulter Vi-Cell analyzing software version 4.0. c) Vi cell data in tabular form as acquired from vi cell counter (n=3) using Vi-Cell analyzer XR through Beckman Coulter Vi-Cell analyzing software version 4.0.

that the gated cells largely comprised of endothelial cells. The expression of CD34, a hematopoietic marker, was low as compared to CD31 and CD146. For negative marker, CD90 or Thy-1 an MSC marker was used which was very low and negligible when evaluated with P1 gated CD146.

Reprogramming

Passaged ECFCs at P2 were brought to transfection and on comparison, the cells brought for reprogramming were of low proliferative potential as discussed by Ferratge et al[28] and after transfection, the reprogrammed cells were grown into N2B27 medium in vitronectin-N coated plate and at day 8, the media was switched to xenofree and serum free complete E8 media. The reprogrammed cells were then picked from 12 well and grown into VTN-N coated 24 wells. The reprogrammed cells showed iPSCs morphology and were then fixed and blocked for immunofluorescence.

Immunofluorescence

Immunofluorescence was performed on iPSCs after immunostaining and the images were acquired at 20X first on bright field then on DAPI filter for nucleus staining and then FITC filter for FITC labelled OCT4 detection on Dmi8 (Leica Microsystems) using LAS software version 4.8 (Leica Microsystems (Switzerland) Ltd). The immunofluorescence confirms that our transfected cells were successfully reprogrammed towards induced pluripotent stem cells as shown in Figure-1 c.

Discussion

The cord blood collected volume was approx. 80-113ml and the processed volume varied from 60-80 ml as shown in Figure 3 (a), cell viability was about 96-97% with total viable cell count of 16 - 46 x 106 as shown in Figure-3 (b), and Figure-1a (i) showing the image acquired by viable cell counter. The ECFC colony appeared in about 5-11 days after isolation (n=3) which were passaged from P1 to P3. The cells were passaged trypsin/EDTA and trypLE Express, the cells were passaged up to P2. The flow cytometry analysis (n=1) of ECFC at P2 shows the positive expression of CD31, CD146 and CD34. The expression of CD31 were high as compared to CD146 whereas CD34 expression was low as compared to CD31 and CD146. The MSCs marker CD90 expression was very low and negligible, All these results showed that our population comprises of bonafide ECFC as shown in Figure-2, the results were similar to those by Yoder and Ingram et al.[5,29]

The cells at P2 were successfully reprogrammed using episomal reprogramming vectors. The reprogrammed ECFC consisted of intermediate colony formation when compared to the characteristics as mentioned by Ferratge[28]. The immunofluorescence (n=1) was positive for OCT4 which is a pluripotent marker and DAPI for nucleus staining. It was observed that the secondary antibody tagged with OCT4 and nucleus, successfully emmitted fluorescent signals. These images confirmed that our cells were successfully reprogrammed towards induced pluripotent stem cells as shown in Figure-1.c (ii) and (iii), reprogramming was successful as stated by Yu et al.[30]

The reprogrammed ECFC can be the source for regenerative pharmacology as they provide a platform for the analysis of compounds and drug screening for angiogenic and vasculogenic potentials. In our experiment we reprogrammed them into iPSCs using episomal vector which has the benefit of non-integration into DNA. Prior to our experiment Ferratge *et al*,[31] reprogrammed ECFC using lentivirus to assess the stemness of their immaturity. The lentivirus has integrative properties which causes mutagenesis and tumorigenesis while episomal vectors have low mutagenic and tumorigenic properties due to transgenic approach. The reprogrammed cells converted to iPS can be the ideal source for regeneration and analysis of a drug or compound in context of regenerative pharmacology which is an emerging advance branch of pharmacology.

Conclusion

The advance avenues in the field of medical sciences open the options for diseases with rare treatment options or that were incurable and could not be treated in the past. The proliferative potential of iPSCs allows them to be used for drug discovery in primary immunodeficiency disorders[32] and it is expected that over the next decade there will be unpredicted and momentous advances in reprogramming for therapeutic agents and in regenerative medicine.[33] In spite of all the innovations, the reprogramming from somatic cells is still an inefficient process[34] but a range of methods have been improved and modified in recent years to improve the efficacy and reliability of reprogramming.[35] Beside all, organoids are the valuable source for safety and efficacious use in drug screening and therapeutics and all these help regenerative medicine towards bedside translation which is the ultimate goal of translational research.

Strength and Limitations

This study provides an avenue to utilize ECFC for drug and toxicological screening for numbers of compound as ECFC have role in angiogenesis and vasculogenesis especially for the anti-tumour drugs. The reprogrammed iPSCs are the source for screening the drugs to provide safer approach in clinical setting. The limitation includes the use of xenogeneic media for the growth of ECFC cell line; the methods are now available for the xenofree growth of ECFCs. The grown ECFC can be analyzed for blood vessel formation on cellular matrix for conducting drug screening.

Acknowledgement: None to declare.

Disclaimer: This research is part of M.Phil. thesis of the first author of the article.

Conflict of Interest: There is no conflict of interest among the authors.

Funding Sources: None to declare.

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