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Effects of Uniaxial Cyclic Strain on Endothelial Progenitor Cells

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Effects of Uniaxial Cyclic Strain on Endothelial Progenitor Cells

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Biomedical Engineering

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1. Abstract

Despite the high prevalence of calcific aortic valve disease (CAVD), the underlying mechanisms of pathogenesis have not been found yet [1]. Therefore, it is extremely important to study CAVD and understand how it develops. For this matter, we decided to study the potential of endothelial progenitor cells (EPCs) for use in tissue-engineered models of heart valves. EPCs were chosen as the cell source of interest for this study due to their high neovascularization potential and use in regenerative medicine and cardiovascular tissue-engineering [2].

In this project, we aimed to engineer the microenvironment of cells that are involved in the formation of heart valves. We hypothesized that cyclic strain induces EPCs to undergo differentiation, which will depend on the applied strain, culture media components and culture duration. EPCs isolated from human umbilical cord blood expressed endothelial cell markers CD31 and vascular endothelial growth factor receptor 2 (VEGFR2), and the progenitor cell marker CD34. The cells did not express the mesenchymal marker α-smooth muscle actin (α-SMA). EPCs showed an endothelial behavior demonstrated by the uptake of acetylated-low density lipoprotein (Dil-Ac-LDL), and a vasculogenic potential demonstrated by tube formation. The cells were subjected to 20% strain rates by utilizing a cyclic uniaxial biostretcher for 7 days and showed a mild expression of α-SMA. Considering these events, EPCs were subjected to 20% strain for longer periods of time (1, 2, and 3 weeks) and showed maintained CD31 expression, no α-SMA expression, and increased CD34 expression suggesting an increased vascular-like behavior after strain.

2. Introduction

Current treatments for valvular heart disease include valve replacement with mechanical prosthetic valves that have a limited ability to grow or remodel. Tissue-engineered cardiac valves are a potential alternative to mechanical prosthetic valves, presenting an optimal valve replacement option for pediatric patients and reducing the risk of immune rejection. The neovascularization potential of Endothelial progenitor cells (EPCs) makes them a reliable cell candidate for regenerative medicine and cardiovascular tissue-engineering [2]. EPCs are circulating cells that have high proliferative and clonogenic potential, and possess the ability to adhere to injured tissue and contribute to postnatal vasculogenesis [3].

Previous studies have shown the ability of EPCs to transform to a mesenchymal phenotype upon exposure to endothelial-mesenchymal transition (Endo-MT) inducers such as transforming growth factor-β (TGF-β) or reduced concentration of serum in cell growth media [4]. Endo-MT is a biologic process by which endothelial cells lose adhesion properties and gain mesenchymal cell phenotype, including enhanced migratory capacity and production of ECM components [5]. Endo-MT is a key process in the cardiovascular embryonic development [6]. Heart valves are composed of valve endothelial cells (VECs) on the surface and valve interstitial cells (VICs) that have a myofibroblast phenotype [6]. VECs have the potential to undergo Endo-MT [6]. Previous studies utilized a custom-made cyclic mechanical stretcher apparatus to apply different levels of strain to VECs and analyzed the effects of a mechanical environment on cell differentiation [6]. The results of these studies suggested that high and low levels of strain affect Endo-MT differently with the low strain load resembling the physiological mechanical stimulus, and high strain load resembling effects observed in disease [6].

In this project, we aimed to successfully isolate EPCs without the use of cell sorting techniques and to differentiate EPCs utilizing uniaxial cyclic strain. We isolated EPCs from human umbilical cord blood using a specialized endothelial medium supplemented with growth factors, studied the effects of using different growth media in cell proliferation, characterized the purity of the colonies obtained, and subjected EPCs to mechanical strain to induce Endo-MT. EPCs were seeded in different media to find the medium that allows for increased cell proliferation. The immunostaining results and the cell proliferation suggested that the use of a specialized endothelial growth media supplemented with growth factors (EGM) increases EPCs proliferation compared to endothelial basal medium (EBM) or Dulbecco's Modified Eagle Medium (DMEM). The growth factors utilized to supplement EGM were: vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which enhance endothelial cell survival, proliferation, and migration; ascorbic acid, which maintains the cobblestone morphology of cells; insulin-like growth factor-1 (IGF-1) to enhance angiogenic and migratory function; epidermal growth factor (EGF) to stimulate cell proliferation and differentiation; hydrocortisone to sensitize the cells to EGF; and heparin to improve long-term stability of growth factors present in the medium [7].

3. Experimental Methods

All protocols were carried out with the approval of the University of Arkansas Institutional Review Board (Approval number 16-04-722).

3.1 Isolation of endothelial progenitor cells:

The isolation was carried out in concordance to the protocol described by Ravishankar et al. as shown in **Figure 1** [8]. Approximately 25 mL of umbilical cord blood was diluted with Hanks Balanced Salt Solution (HBSS) at a 1:1 concentration. 20 mL of density gradient medium and the diluted blood were layered in the same tube and centrifuged without brakes at 800xg for 30 min to separate the blood components in different layers as shown in **Figure 1**. The plasma layer was pipetted out carefully and properly disposed of, enabling the pipette to reach the mononuclear cells (MNCs) layer and collect it into a different tube. EGM was used to resuspend the pellet of MNCs, and the cells were seeded in 6-well plates pre-coated with 2 mL of rat tail I collagen solution.

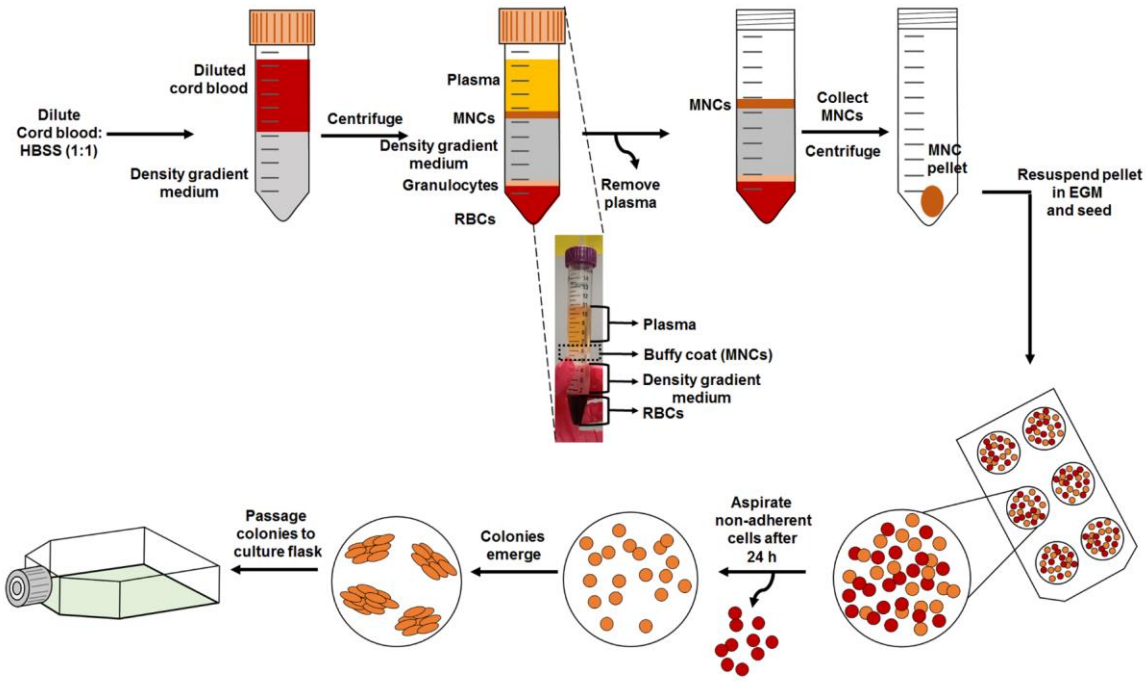

Figure 1: Schematic of endothelial progenitor cell isolation. Blood components were separated, and the mononuclear cells (MNCs) were collected and seeded in collagen-coated plates.

The medium was aspirated after 24 h, and the wells were washed once with EGM. For 7 days, the plate was refreshed with EGM every day. After 7 days, EGM was changed every other day. The outgrowth of colonies started to appear in culture after 5 and 9 days of seeding MNCs as observed in **Figure 2A**. Spindle-shaped cell morphology is observed in the early stages as shown in **Figure 2A-B** which at later stages, progressed into cobblestone-like morphology (**Figure 2C-D)**.

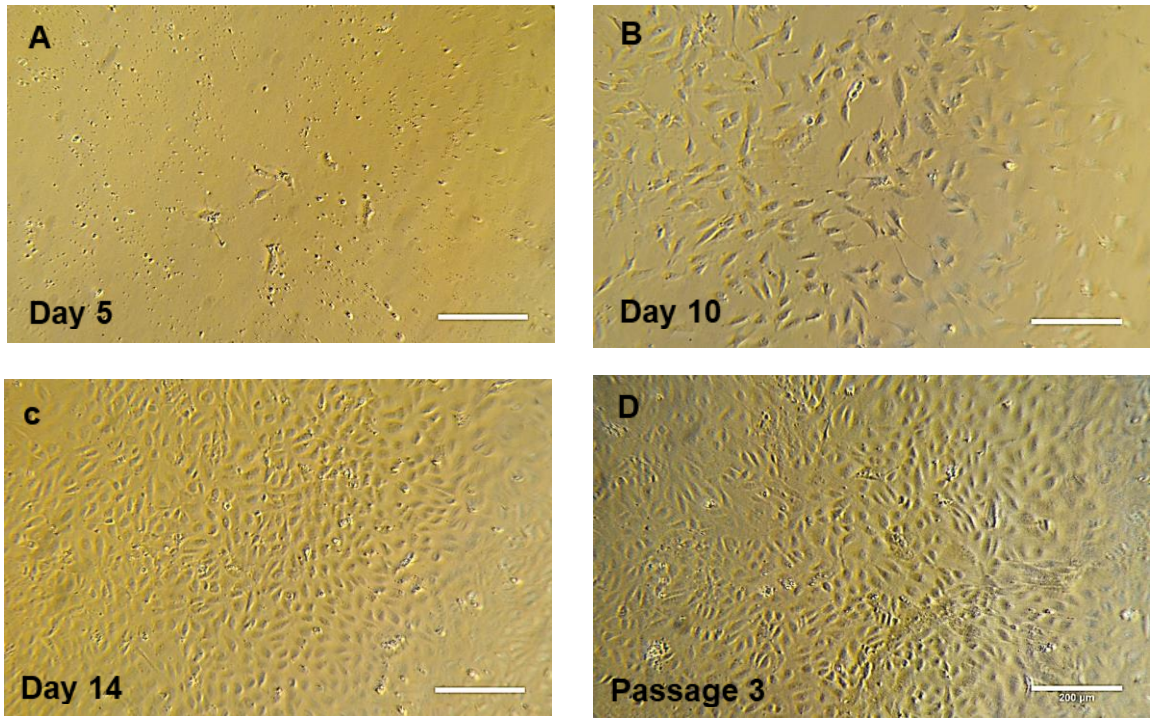

Figure 2. Cell colony progression over time. (A-B) Representative bright-field microscope images of EPC colony progression on days 5 and 10 presenting a spindle-shaped morphology. (C-D) Representative bright-field microscope images of EPCs at later stages presenting a

3.2 Characterization of endothelial progenitor cells:

3.2.1 Western blotting of EPC lysates

Isolated EPCs were characterized for surface markers via western blotting. Approximately 500,000 cells were lysed, and their proteins were collected. Proteins were quantified using bicinchoninic acid (BCA) assay [8-11]. 5 µg of protein were tested for expression of CD31, CD34, vascular endothelial growth factor receptor 2 (VEGFR2) and α-smooth muscle actin (α-SMA) using standard procedures for western blotting [8-11]. Human umbilical vein endothelial cells (HUVEC) was used as a positive control for CD31, CD34, and VEGFR2. Valvular interstitial cells (VIC) were used as a positive control for α-SMA and α-tubulin was used as a loading control.

3.2.2 Immunofluorescence of EPCs

EPCs were seeded in Type-I Collagen coated coverslips. After 24 hours, the coverslips were fixed with methanol and immunostained for CD31, CD34, VEGFR2, and α-SMA using standard procedures [8-11].

3.2.3 Acetylated-low density lipoprotein (Dil-Ac-LDL) uptake assay

EPCs were seeded on Type-I Collagen coated coverslips and treated with Dil-Ac-LDL at 10µg/ml concentration. Following a five-hour incubation, the coverslips were fixed in 4% PFA, mounted on a glass slide and imaged.

3.2.4 Matrigel Assay

Matrigel was diluted at 1:1 with EGM and 30µL per well of diluted Matrigel was added to a 96-well plate and incubated for 30 min. $1x10^5$ cells were seeded per well on the Matrigel-coated 96 well plate. After 12 hours, samples were imaged under a brightfield microscope.

3.3 Uniaxial cyclic strain of EPCs

EPCs were seeded onto a PDMS elastomeric membrane and subjected to strain magnitudes of 0% (no strain control), and 20% (pathological strain) in a custom-built uniaxial bio-stretcher (**Figure 3**) at 1 Hz for 1, 3 and 7 days in EGM. The next set of EPCs were cultured in two different medium conditions- EGM and EGM without FGF and exposed to the same strain conditions described above for 1, 2 and 3 weeks.

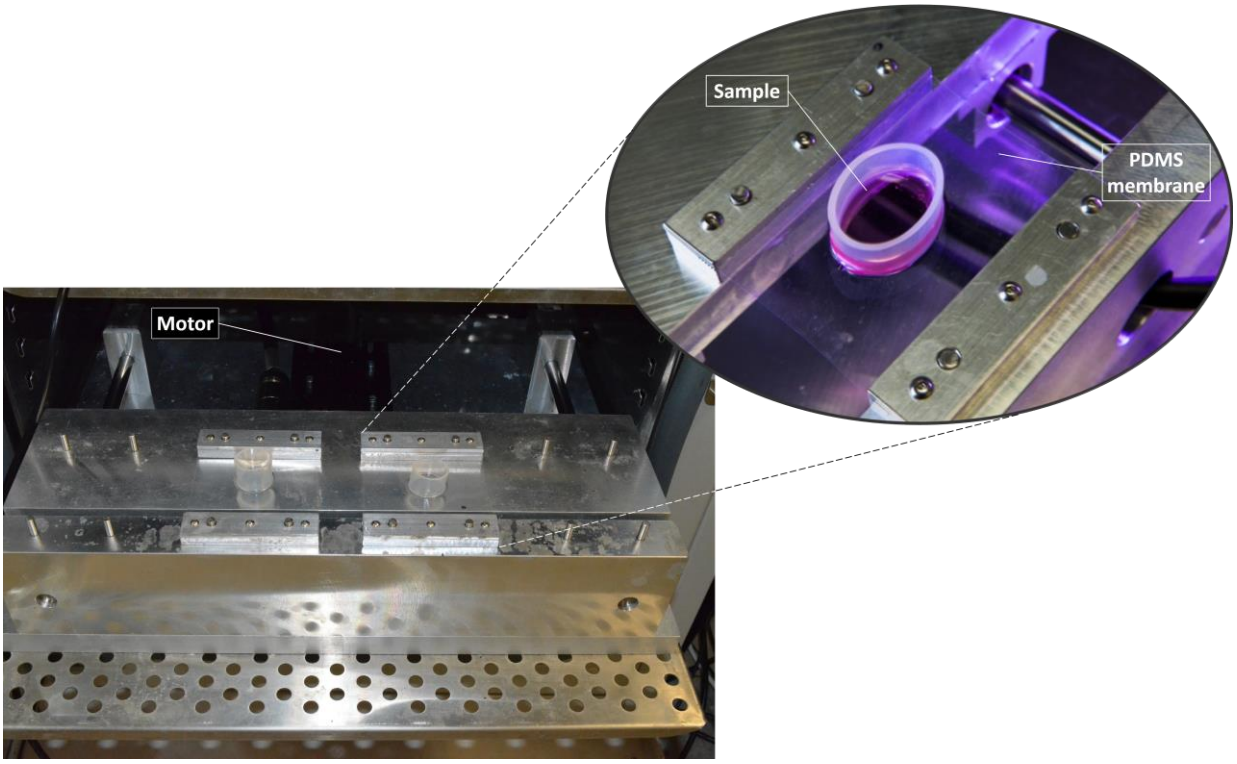

Figure 3. Photograph of two PDMS elastomeric chambers mounted on the uniaxial cyclic biostretcher, a custom-built apparatus that subjects samples to adjustable amounts of strain. Zoom-in view of the PDMS elastomeric membrane with the sample placed in the silicon ring.

3.4 Characterization of markers after cyclic strain

3.4.1 Western blotting of EPCs after cyclic strain

After subjecting the cells to cyclic strain, proteins were collected from the elastomeric membranes using the same western blotting procedure described above.

3.4.2 Immunofluorescence of EPCs after cyclic strain

Stretched EPCs were fixed and immunostained for nuclei using 4,6-diamidino-2-phenylindole (DAPI), and for acting filaments with Alexa Fluor 488-phalloidin following standard procedures [8-11].

4. Results and Discussion

4.1 Characterization of isolated endothelial progenitor cells

The morphology of the isolated EPCs progressed from spindle-shaped (**Figure 2A-B**) in the early stages to cobblestone morphology (**Figure 2C-D**) at later stages in culture. Various research groups have labeled EPCs differently, including late endothelial progenitor cells [12],

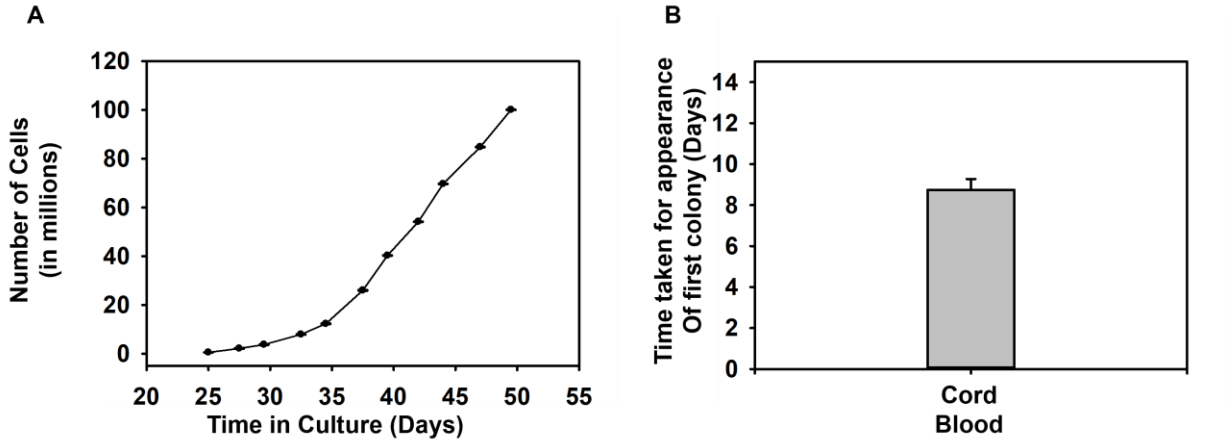

Figure 4. Growth curves of isolated EPCs. (A) Number of cells growing in culture over time. Each data point represents the cell number harvested during each passage (P0-P10) (n = 4). (B) Time taken for the first EPC colony to appear in the 6-well plate (n = 4). The error bars denote the standard error of the mean (SEM). No statistical significance was found using one-way ANOVA.

endothelial colony forming cells [13], or endothelial progenitor cells [14]. However, all these cells perform the same functions and express similar cell surface markers. The EPCs isolated in this project showed a typical high proliferative potential (**Figure 4**) [13,15].

Isolated EPCs seeded in different media demonstrated that the use of a specialized EGM increases EPCs proliferation compared to EBM or DMEM (**Figure 5**). Cells cultured in EGM showed increased proliferation due to the presence of growth factors described before.

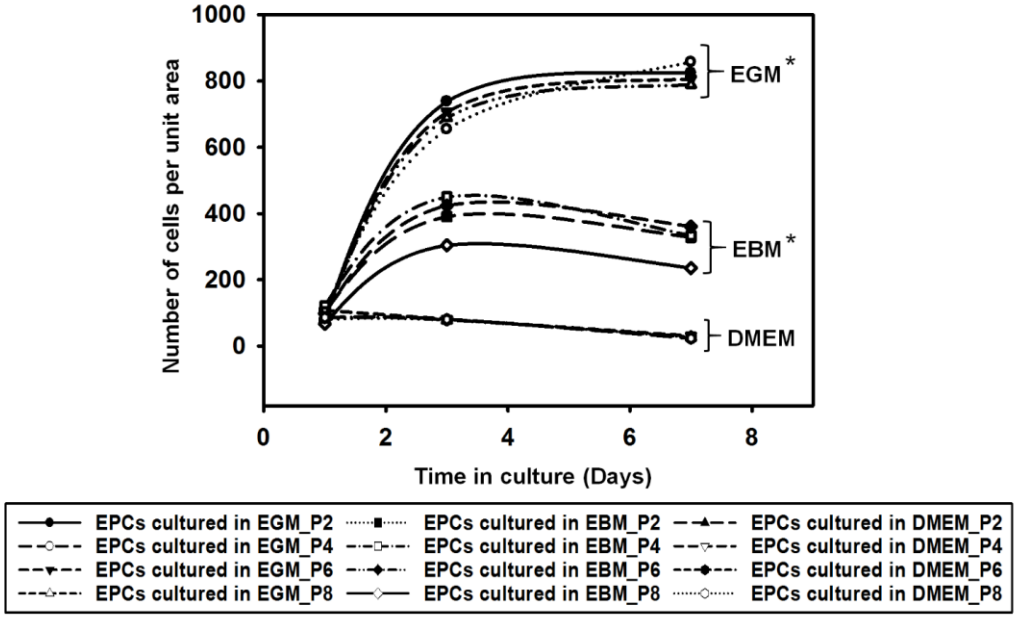

Figure 5. Growth curve of EPCs cultured in EGM, EBM, and DMEM. EGM facilitated higher cell growth compared to EBM and DMEM. The data showed statistical significance with $p<0.01$ using One-way ANOVA. (n=2)

The number of cells per unit area per well over time is shown for each media condition in **Figure 5**. EPCs seeded in EGM presented a steady growth rate increase, while cells in EBM showed a slower growth rate that starts to decrease after day 3. This suggests that EPCs have the ability to

survive in EBM, but depend on the growth factors present in EGM for maintaining proliferation over time. EPCs cultured in DMEM decreased over time, suggesting that it is not suitable for EPCs survival and proliferation.

The same results were supported by immunostaining of isolated EPCs in each media condition (**Figure 6, 7 and 8**). Immunostaining results demonstrated that the isolated EPCs were not contaminated with other types of cells and that the cells expressed the endothelial markers CD31 and VEGFR2, the vascular marker CD34, and did not express the mesenchymal marker α-SMA.

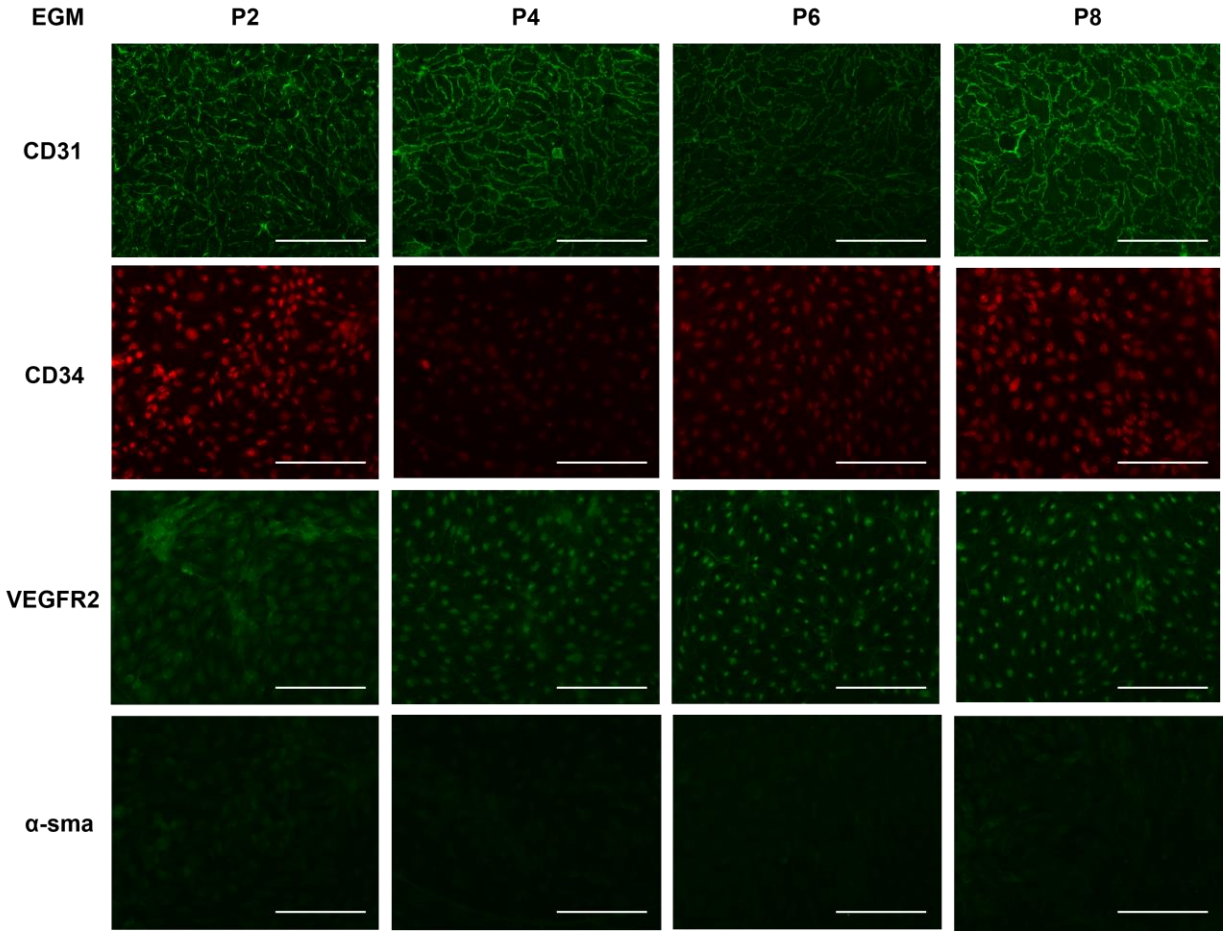

Figure 6. Immunostaining of EPCs in EGM. Representative images of EPCs cultured in EGM for 7 days and immunostained at various passages for CD31, CD34, VEGFR2, and α-SMA. Scale bar: 100 µm.

Additionally, the expression of CD31 and CD34 decreases over time from passage to passage (**Figure 6**). Comparing **Figure 6** with **Figures 7 and 8**, EPCs cultured in EGM showed a qualitative increase in CD31 expression than EPCs seeded in EBM and DMEM.

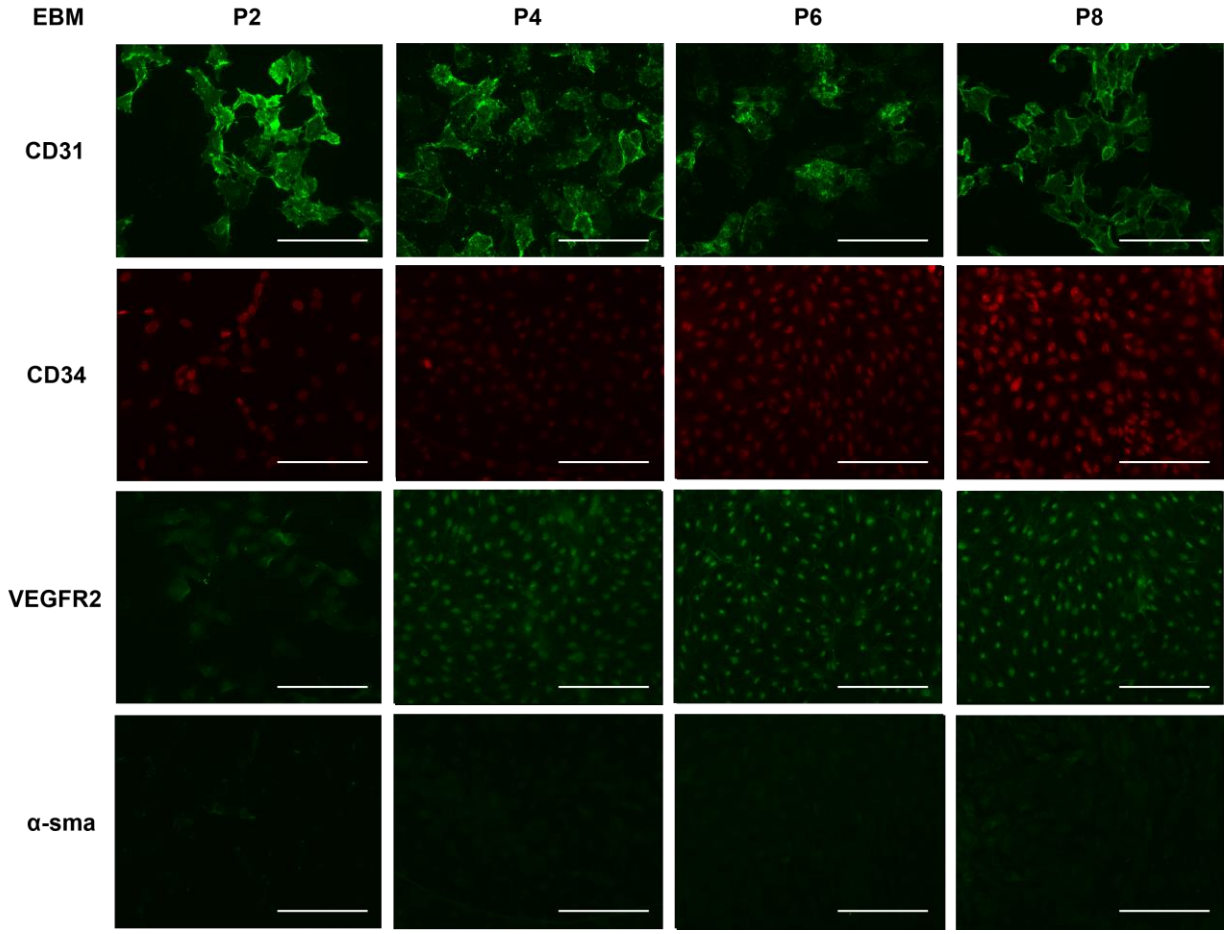

Figure 7. Immunostaining of EPCs in EBM. Representative images of EPCs cultured in EBM for 7 days and immunostained at various passages for CD31, CD34, VEGFR2, and α-SMA. Scale bar: 100 µm.

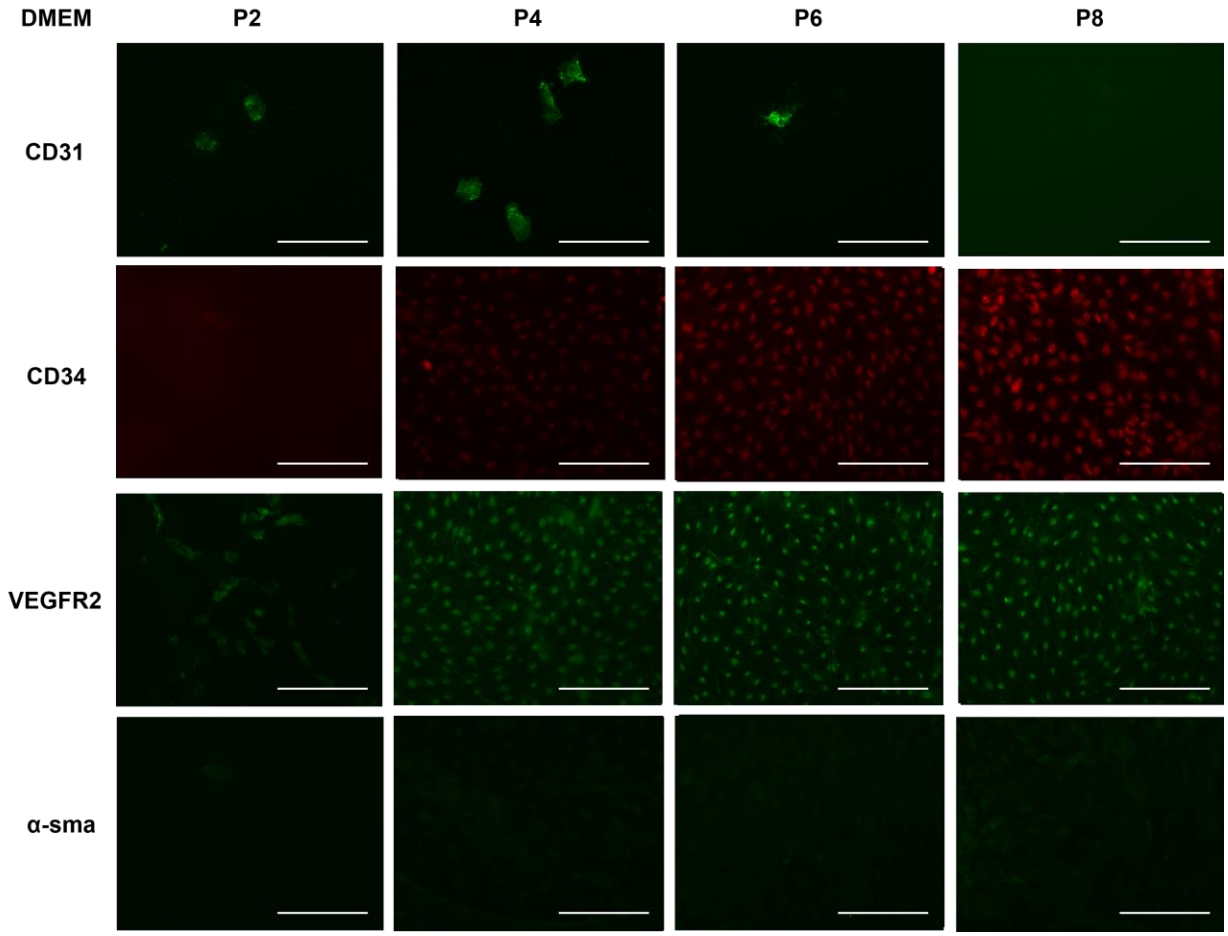

Figure 8. Immunostaining of EPCs in DMEM. Representative images of EPCs cultured in DMEM for 7 days and immunostained at various passages for CD31, CD34, VEGFR2, and α-SMA. Scale bar: 100 µm.

In the same way, western blotting results corroborated the expression of CD31, CD34, and VEGFR2 on the isolated EPCs lysates compared to the positive control, HUVEC (**Figure 9**). Analogous to previous studies, CD31, CD34 and VEGFR2 expression decreased in higher passages [12, 14, 16]. α-SMA was not expressed in EPCs, verifying that this type of cell is not mesenchymal in nature (**Figure 9**).

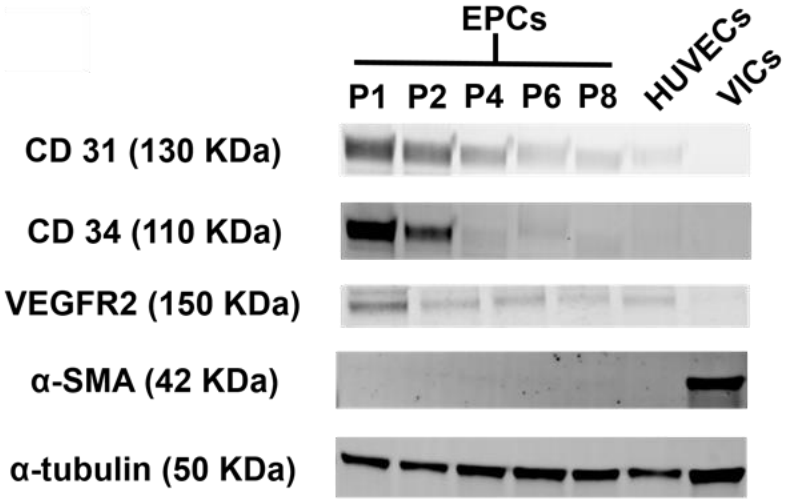

Figure 9. Western blotting of isolated EPCs. Human Umbilical Vein Endothelial Cell (HUVEC) lysates were used as a positive control for CD31 and CD34. Valve Interstitial Cell (VIC) lysates were used as a positive control for α-SMA.

To further support our isolation, we carried out functional assays like Dil-Acetylated LDL uptake and matrigel assay [12, 13]. The results of the Dil-Ac-LDL uptake assay showed that EPCs metabolized Dil-Ac-LDL compared to VICs (negative control) that showed no uptake of Dil-Ac-LDL (**Figure 10A**). Dil-Ac-LDL uptake capacity is a characteristic of the endothelial-like behavior of EPCs.

Matrigel assay results showed that EPCs formed tube-like structures owing to its vasculogenic potential whereas VICs did not form these structures and tended to clump into nodules in the Matrigel assay (**Figure 10B**).

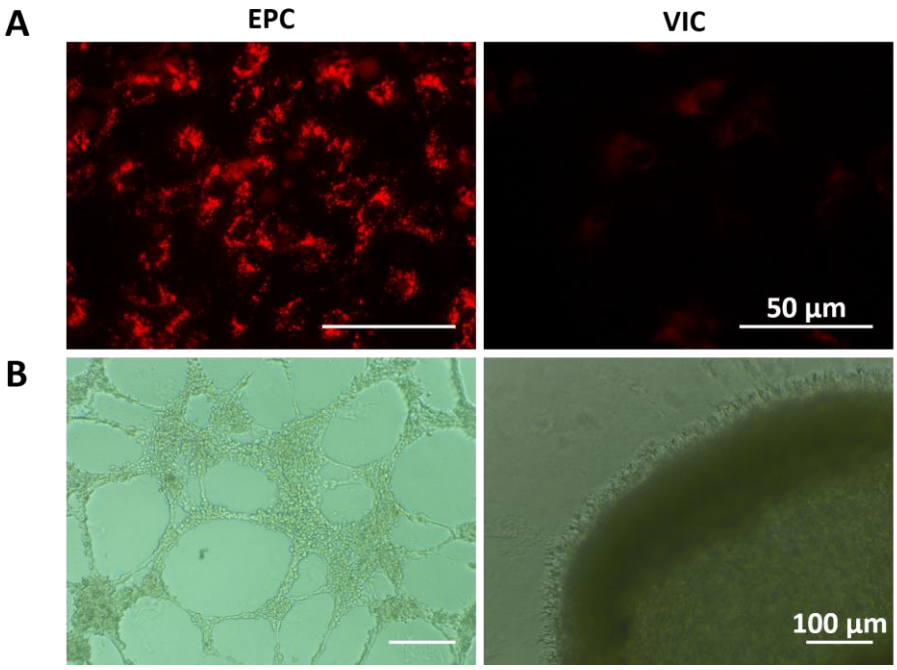

Figure 10. (A) Dil-Ac-LDL uptake assay. Representative fluorescent microscope images of EPCs (on the left) after Dil-Ac-LDL uptake compared to VICs (on the right) that showed no uptake of Dil-Ac-LDL. (B) Matrigel assay. EPCs showed tube formation, whereas the VICs (negative control), did not show tube formation.

4.2 Cyclic strain altered EPC morphology and phenotype

After the characterization of EPCs, the cells were subjected to cyclic strain in a custom-built biostretcher shown in **Figure 3**. The strain values as a function of time were plotted in **Figure 11A**, demonstrating a 20% applied strain. The actin orientation order parameter (OOP) was shown to be significantly increased in EPCs subjected to strain, compared to the 0% strain control (**Figure 11B**). Uniaxial cyclic strain in the direction shown by the white arrow in **Figure 11C**, caused EPCs to align in the opposite direction to the applied strain.

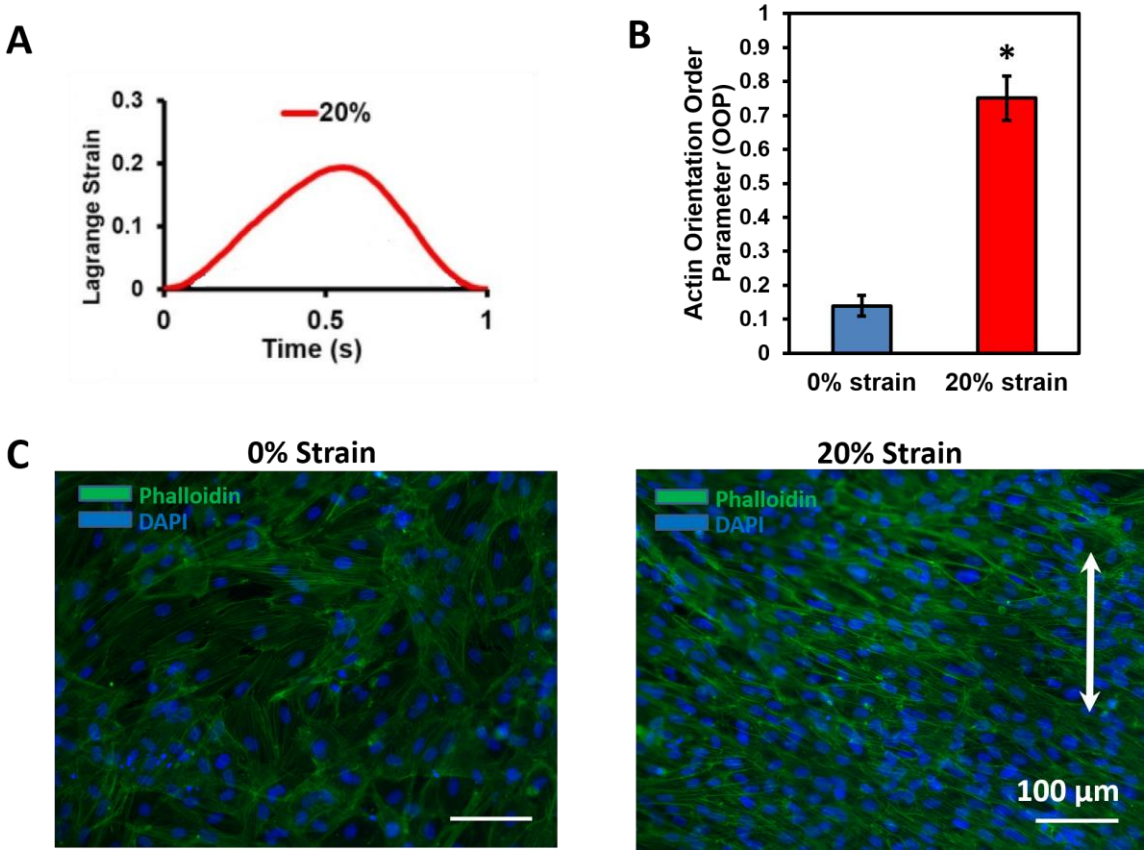

Figure 11. Effects of cyclic strain on EPC morphology. (A) Mean temporal strain waveforms imposed on the membrane. (B) Actin OOP measurement where 0 denotes random orientation and 1 means completely aligned. (* shows statistical significance with $p<0.05$) (C) Immunostained images of EPCs qualitatively showing the alignment of cells. The arrow denotes the direction of applied cyclic strain.

Initially, we analyzed the expression of CD31, CD34, and α-SMA on EPCs that were subjected to strain for 1, 3, and 7 days. The western blotting results showed decreased expression of progenitor marker CD34 and a mild increase in expression of the mesenchymal marker α-SMA after seven days (**Figure 12**). These results suggested that Endo-MT could have started taking place approximately on day 7.

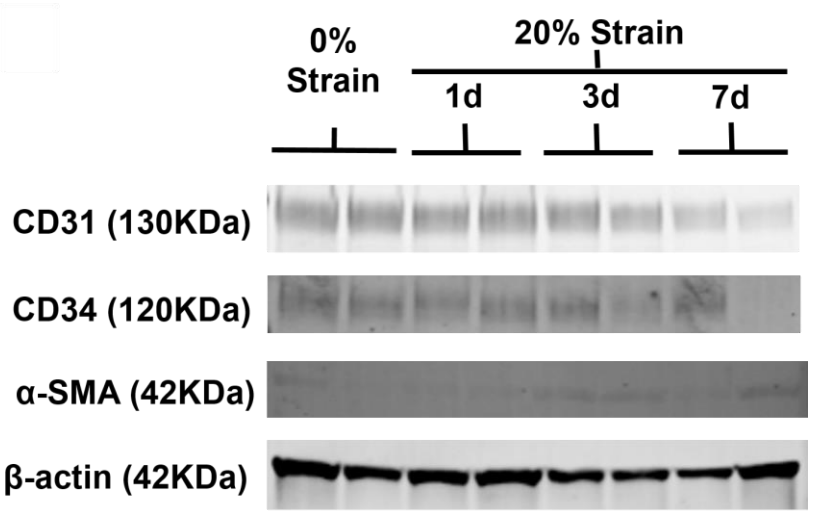

Figure 12. Effects of cyclic strain on phenotype of EPCs up to 7 days. Western blotting of cells collected upon completion of uniaxial cyclic stretch after 1, 3, and 7 days. EPC from Day 0 that was used for seeding, HUVEC and VIC lysates were used as controls.

Since the mesenchymal marker α-SMA showed increased expression after 7 days, we decided to subject the cells to longer periods of cyclic strain (1, 2, and 3 weeks) expecting to observe an increased α-SMA expression. Additionally, the EPCs were seeded in EGM and EGM without FGF to analyze the effects of the growth factor on EPCs phenotype. We chose these media conditions because of the potential role of FGF in maintaining EPC phenotype [17]. The western blotting results showed that CD34 expression increases over time, CD31 expression is

maintained regardless of the period of strain, and α-SMA expression does not increase upon exposure to cyclic strain (**Figure 13**). These results suggest that EPC behavior becomes more vascular-like while maintaining their endothelial phenotype and that Endo-MT does not take place after mechanical stimulation. Additionally, **Figure 13** shows that FGF allows to maintain CD34 expression suggesting that without FGF cells behave in a more endothelial-like manner.

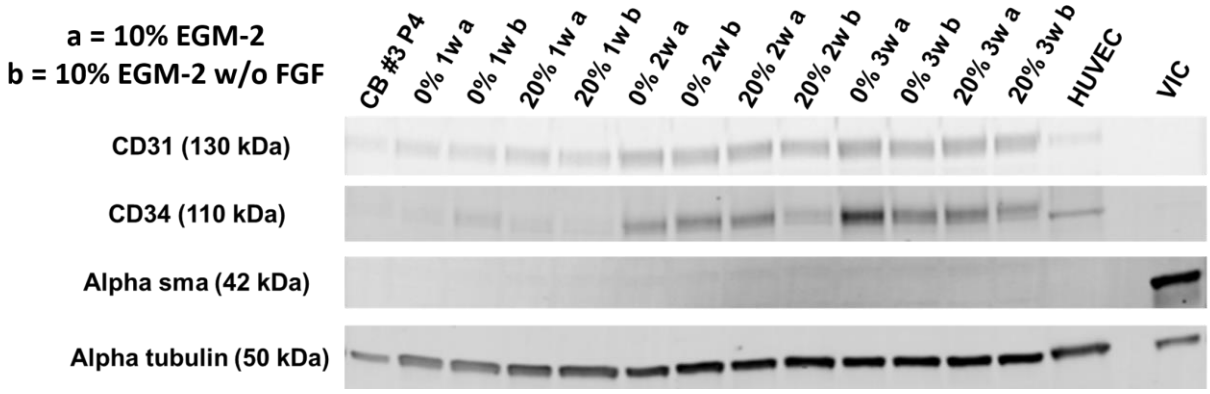

Figure 13. Effects of cyclic strain and FGF on phenotype of EPCs. Western blotting of EPCs subjected to cyclic strain at 0% and 20% for 1, 2 and 3-week time periods.

5. Conclusions and Future Work

In conclusion, EPCs were successfully isolated from human cord blood in a specialized media supplemented by growth factors that allow for high levels of proliferation and survival. Isolated EPCs expressed CD31 and VEGFR2, indicating their endothelial phenotype. EPCs also expressed CD34, suggesting an early hematopoietic and vascular-associated phenotype. EPCs did not express α-SMA, a mesenchymal phenotypic marker. Also, EPCs demonstrated Dil-Ac-LDL uptake and tube-like formation.

After subjecting EPCs to uniaxial cyclic strain, we observed that the cells preferentially oriented perpendicular to the direction of applied strain. EPCs showed an increase in the expression of

CD34 over time under conditions treated with EGM-2. Also, CD31 expression was unchanged suggesting that the cells behaved more vascular-like while maintaining their endothelial phenotype. The cells did not express any α-SMA over the study duration suggesting that Endo-MT did not take place. In the future, we plan on testing different growth factors and their synergic effects with uniaxial cyclic strain over longer culture durations.

All these observations lead us to conclude that EPCs show a phenotypic change towards a vascular phenotype when subjected to uniaxial cyclic strain. This project could also be complemented with the analysis of EPC-secreted components upon mechanical stimulation or the use of a biaxial biostretcher. Future work could also deepen into using other variations of EGM such as decreased percentages of fetal bovine serum in media to induce differentiation of EPCs into a mesenchymal type. Further studies will look at using EPCs as a source to build tissue-engineered cardiovascular constructs.

6. Acknowledgements

Foremost, I would like to express my sincere gratitude to my research mentor Dr. Kartik Balachandran for the continuous support of my career development and research projects, for his insightful comments and feedback, and for the opportunity to participate in research and to join his lab. I am especially grateful to my supervisor Prashanth Ravishankar, for his patience, enthusiasm, and motivation to keep expanding my skills and knowledge. Prashanth guided me not only in the development of research skills, but also in the thought process of designing experiments and generating knowledge. I could not have imagined having a better group of work for my undergraduate research experience. I am grateful to all of those with whom I have had the pleasure to work during this research project.

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I will be forever grateful to my family for giving me the opportunity to be part of the University of Arkansas and motivating me to follow my dreams.

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