Co-administration of First Trimester Umbilical Cord-Derived Perivascular Cells (FTM HUCPVCs) with Endothelial Progenitor Cells (EPCs) Leads to Enhanced Angiogenesis, both *in vitro* and *in vivo*, Compared to either Cell Type alone

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INTRODUCTION

FTM HUCPVCs are a novel, young source of mesenchymal stromal cells (MSCs) isolated from the perivascular region of first trimester umbilical cords[1]. As they support vasculature in their tissue origin, we explored their beneficial angiogenic effect *in vitro* and *in vivo*. We have previously shown that FTM HUCPVCs have superior angiogenic properties compared to older sources of human MSCs.

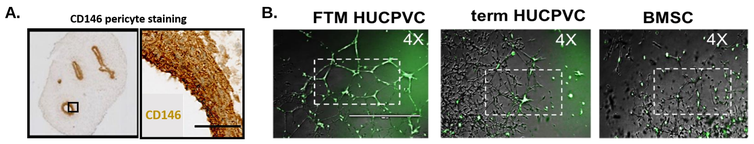

Figure 1: FTM HUCPVCs can be identified in the perivascular region of umbilical cords using pericyte marker CD146 (A). Assessment of MSC homing and network integration using aortic ring assay (B).

In our novel experimental approach, we applied endothelial progenitor cells (EPCs), the building blocks for neovasculature, in combination with FTM HUCPVCs to achieve a greater angiogenesis through the development of sustainable endothelial networks and functional blood vessel formation using *in vitro* and *in vivo* angiogenesis assays.

Hypothesis

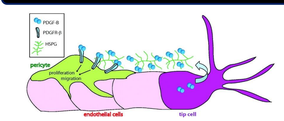

Figure 2: Endothelial–pericyte interactions in microvessels[2].

Hypothesis: A combined cell therapy approach using FTM HUCPVCs as "vasculature supportive cells" and EPCS as "building blocks" will lead to more mature and sustained vascularization in *in vitro* and *in vivo* models of angiogenesis.

MATERIALS AND METHODS

In vitro Angiogenesis Tube Formation Assay

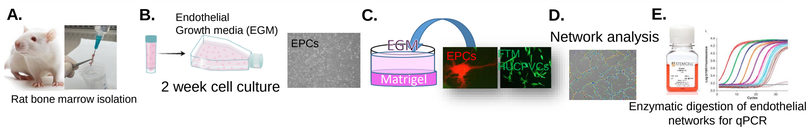

Figure 3: EPCs were isolated from 8 week old male SD rats. Femur and tibia were flushed with HBSS and filtered using 70um filter mesh **(A).** Bone marrow suspensions were plated on T75 flasks and cultured with endothelial growth media-complete factor cocktail (Lonza) **(B).** Following two weeks of EPC culture, pre-stained FTM HUCPVCs expanded in α-mem 5% human platelet lysate (Compass Biomedical PLUS™) and EPCs and were plated together on Matrigel™ -coated plates (1:2, respectively) **(C).** Images of endothelial networks were quantified at Day 1 and 3 using ImageJ™**(D)**. At endpoint analysis, endothelial networks were digested and processed for angiogenesis-specific qPCR arrays (Qiagen) **(E)**.

In vivo Matrigel Plug Angiogenesis Assay

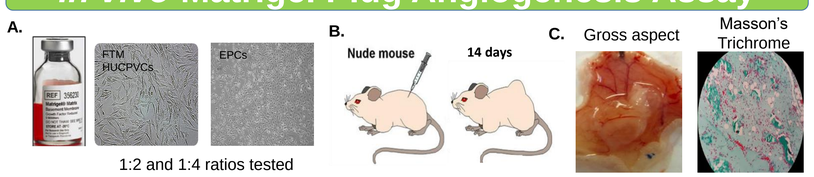

Figure 4: Set-up of Matrigel Plug Assay: Suspension of FTM HUCPVCs and rat EPCs with Matrigel. 1:2 and 1:4 ratios were tested respectively **(A)**. Subcutaneous co-injection of MSCs, EPCs and Matrigel were injected in 8-week nude mice for 14 days **(B)**. Matrigel plugs were isolated with surrounding skin tissue to quantify recruitment of neighboring vasculature. Following, Matrigel plugs were removed from skin sections and fixed using 10% Formalin. Masson's trichrome staining identified perfused vasculature (red) and degree of ECM remodeling (turquoise) allowing quantification of perfused blood vessels and degree of ECM remodeling **(C)**.

RESULTS

FTM HUCPVCs cultured with EPCs promote significant tube formation by supporting developing vasculature

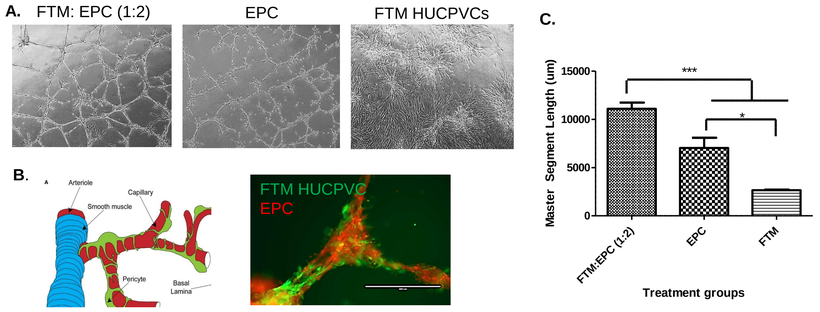

Figure 5: Bright field microscopy images show large endothelial networks with closed loops when FTM HUCPVCs and EPCs are co-cultured together on Matrigel. Tip cells can be identified emerging from vessel-like structures. EPCs alone on Matrigel form network like structures with discontinuous networks, while FTM HUCPVCs developed aggregate-like structures with large cell proliferation areas **(A)**. Florescence microscopy on pre-labelled FTM HUCPVCs and EPCs reveals typical interactions of pericytes and endothelial cell[2] **(B)**. Quantification of network segment lengths shows greater network development in FTM:EPC co-cultures versus either cell type alone **(C)**. Statistics completed using one-way Anova. N=3 * (p<0.05), *** p<0.001

FTM HUCPVCs upregulate key factors of angiogenesis initiation and maintenance when co-cultured with EPCs

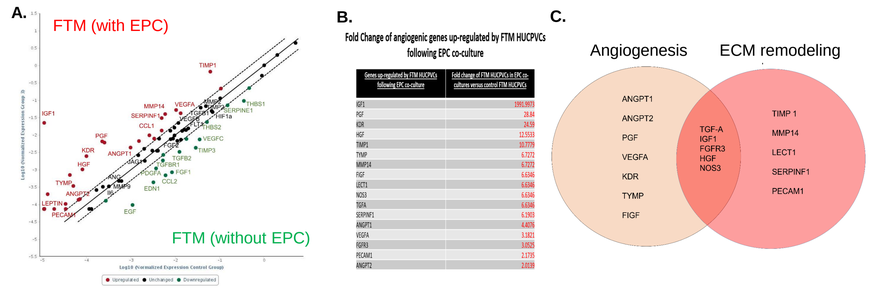

Figure 6: Angiogenesis and extracellular matrix (ECM) modulating factor gene expression profile in FTM HUCPVCs co-cultured with EPCs, 7 days following co-culture versus FTM HUCPVCs control **(A)**. FTM HUCPVCs co-cultured with EPCs upregulate key angiogenic growth factors (ANGPT1, ANGPT2, PGF, VEGFA, KDR, TYMP. FIGF), ECM remodeling factors (TIMP1, MMP14, LECT1, SERPINF1 and PECAM1) and several factors involved in both processes (TGFa, IGF1, FGFR3, HGF and NOS3). Critical angiogenic factors are highly expressed in both FTM HUCPVCs from co-cultures and control conditions (TGFB,VEGFB, FLT1,MMP2,9,ANG,IL6 (black dots in dot plot) **(A)**. Key factors up-regulated are listed with fold changes **(B)** and grouped using Venn diagram **(C).** Ct>28 considered negligible. N=2 p<0.05

Co-injection of FTM HUCPVCs and EPCs improve vascular recruitment to Matrigel Plugs compared to EPC alone

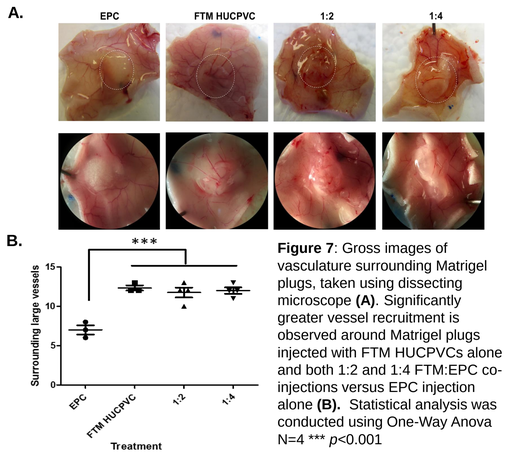

Figure 7: Gross images of vasculature surrounding Matrigel plugs, taken using dissecting microscope **(A)**. Significantly greater vessel recruitment is observed around Matrigel plugs injected with FTM HUCPVCs alone and both 1:2 and 1:4 FTM:EPC co-injections versus EPC injection alone **(B).** Statistical analysis was conducted using One-Way Anova N=4 *** p<0.001

FTM HUCPVCs and EPCs co-injected significantly improve Matrigel Plug vascular perfusion

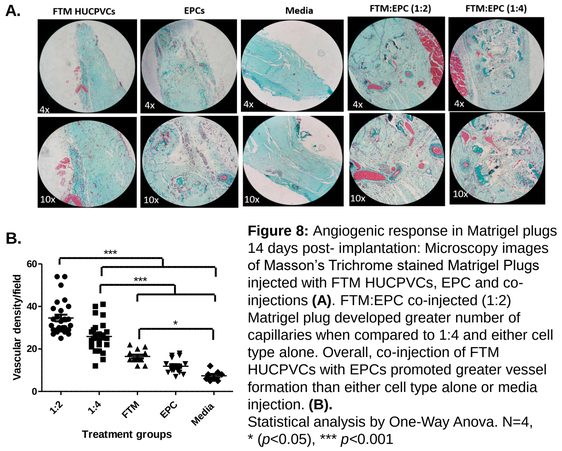

Figure 8: Angiogenic response in Matrigel plugs 14 days post- implantation: Microscopy images of Masson's Trichrome stained Matrigel Plugs injected with FTM HUCPVCs, EPC and co-injections **(A)**. FTM:EPC co-injected (1:2) Matrigel plug developed greater number of capillaries when compared to 1:4 and either cell type alone. Overall, co-injection of FTM HUCPVCs with EPCs promoted greater vessel formation than either cell type alone or media injection. **(B).**
Statistical analysis by One-Way Anova. N=4, * (p<0.05), *** p<0.001

ECM remodelling activity is greater with FTM HUCPVC and EPC co-injection

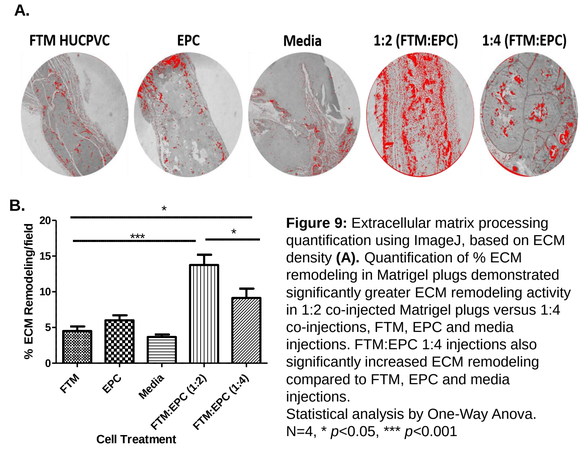

Figure 9: Extracellular matrix processing quantification using ImageJ, based on ECM density **(A).** Quantification of % ECM remodeling in Matrigel plugs demonstrated significantly greater ECM remodeling activity in 1:2 co-injected Matrigel plugs versus 1:4 co-injections, FTM, EPC and media injections. FTM:EPC 1:4 injections also significantly increased ECM remodeling compared to FTM, EPC and media injections.
Statistical analysis by One-Way Anova. N=4, * p<0.05, *** p<0.001

CONCLUSION(S)

- **FTM HUCPVCs co-cultured with EPCs significantly improve *in vitro* tube formation. FTM HUCPVCs provide physical support for EPCs to promote structured and stable networks.**
- **FTM HUCPVCs up-regulate key angiogenesis initiator and maintenance factor genes along with ECM remodeling factors for favorable tube formation.**
- **Matrigel Plugs show significantly greater vascular recruitment and perfusion of Matrigel plugs and increased ECM remodeling when co-injected with FTM HUCPVCs and EPCs compared to single cell types.**
- **1:2 ratio of co-culture and co-injection of FTM HUCPVCs and EPCs results in optimal angiogenic response**

These results highlight the superior nature of a co-administration cell therapy for vascular regenerative treatment of ischemia-associated pathologies.

ACKNOWLEDGEMENTS

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