

# Scaffolds Covalently Immobilized with VEGF and Angiopoietin-1 to Promote Angiogenesis In Engineered Cardiac Tissues

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## Introduction:

Cardiovascular diseases have been the main cause of death in Canada for the past 25 years, and accounted for more than 30% of deaths in 2004 [1]. Myocardium infarction causes irreversible damage to the heart, and the heart becomes incapable of regeneration due to the non-proliferative nature of terminally differentiated adult cardiomyocytes. As a result, there is motivation to engineer cardiac tissue patches *in vitro* by culturing cardiac cells in a scaffold matrix, after which these patches can be grafted into the diseased heart to regenerate the injured myocardium.

One of the recent challenges in cardiac tissue engineering is that functional blood supply is needed for constructs larger than 100-200 microns in thickness in order to provide sufficient oxygen and nutrients. Oxygen transport only occurs up to a distance of 100 $\mu$ m under normal medium diffusion [2]. Cell density and viability is compromised under limited oxygen supply [3]. One solution is to design a physiologically interactive replacement consisting functional blood vessels for the injured vascular tissue. By inducing vascularization within the engineered tissue *in vitro*, limited transport capacity of oxygen and nutrients into the tissue can be overcome, thus improving its survival both *in vitro* and *in vivo* [4].

Neovascularization requires coordination of multiple endothelial growth factors, receptors and intracellular signalling pathways. Amongst these angiogenic growth factors, vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) have been shown to be the most important regulators of blood vessel formation. Past studies have shown that VEGF promotes the formation of new capillary vessels, while Ang1 induces the maturation and stabilization of new vessel networks, suggesting a complementary relationship between these two angiogenic factors [5].

In this study, we immobilized VEGF and Ang1 in collagen scaffolds to protect growth factors against cellular inactivation and digestion, and to allow highly sustained and localized activity. We hypothesize that VEGF and Ang1, when covalently immobilized in collagen scaffolds, work together to first form new vessels, and then to stabilize these vessels.

## Materials and Methods:

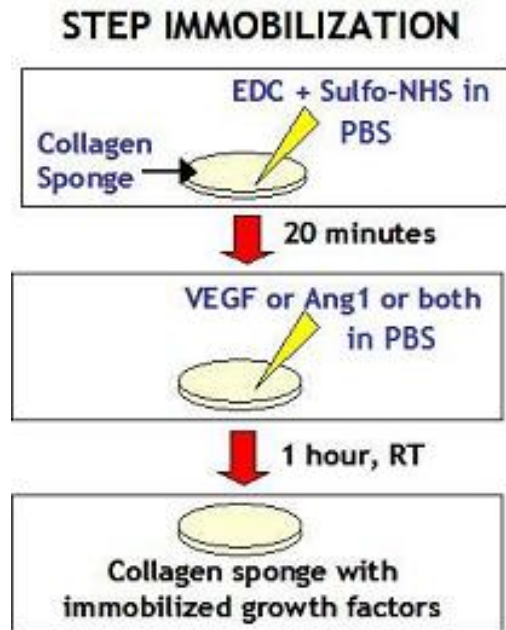
A metal borer was used to cut circular collagen scaffolds with diameter of 7mm and thickness of 2mm from a sheet of porous collagen sponge (Ultrafoam collagen sponge, Davol, 1050050). Both mouse recombinant VEGF-165 (Cell Sciences, CRV014B) and human

recombinant Ang1 (R&D Systems, 923-AN-025) were immobilized into the scaffolds using EDC chemistry (**Figure 1A**). EDC chemistry was performed by immersing the collagen scaffolds into 150 $\mu$ L sterile filtered solution of EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma, E7750) and Sulfo-NHS (N-hydroxysulfosuccinimide, Pierce Chemicals, 24510) in a 96-well plate (EDC/sulfo-NHS concentrations of 24mg/60mg per 1mL 1M PBS). The activation of the collagen scaffolds was allowed to proceed for 20 minutes at room temperature. The scaffolds were then removed from the solution, and immersed into 100 $\mu$ L solution of a) 1 $\mu$ g/mL VEGF (VEGF+PBS), b) 1 $\mu$ g/mL Ang1 (Ang1+PBS), c) 0.5 $\mu$ g/mL VEGF and 0.5 $\mu$ g/mL Ang1 (1/2VEGF+1/2Ang1), or d) 1 $\mu$ g/mL VEGF and 1 $\mu$ g/mL Ang1 (VEGF+Ang1) in PBS. The reaction was allowed to proceed for 1 hour at room temperature. For control samples (PBS control), collagen sponges were immersed in PBS at room temperature. Afterwards, the scaffolds were immersed in fresh PBS for 8 times to wash away the uncrosslinked VEGF and/or Ang1, EDC and sulfo-NHS. After washing in PBS, the scaffolds were incubated in culture medium for 30 minutes and dried on autoclaved Kim-wipes prior to cell seeding.

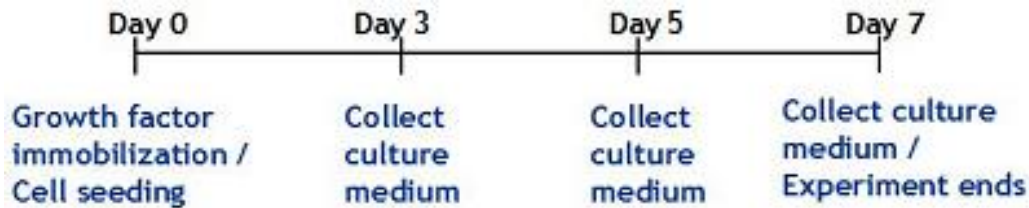
For *in vitro* studies, the collagen scaffolds were transferred to a clean 24-well plate after scaffold preparation. 50000 H5V endothelial cells were seeded onto the scaffolds in 10 $\mu$ L culture medium. The scaffolds were incubated for 40 minutes at 37°C for cells to attach. After incubation, 1mL fresh culture medium was added to each well. The samples were cultured for 3 or 7 days with a 100% change and collection of culture medium on Day 3, Day 5 and Day 7 (**Figure 1B**). Collected culture medium was stored at -20°C for further lactate and glucose assays. The samples were analyzed for final cell number by XTT assay, lactate production rate by lactate assay, glucose consumption rate by glucose assay, and cell density at various heights of the scaffold by cryosectioning and DAPI staining. The effective VEGF and Ang1 concentrations were quantified by ELISA.

For preliminary *in vivo* studies, the collagen scaffolds immobilized with solutions of a) 0 $\mu$ g/mL VEGF (control), b) 0.5 $\mu$ g/mL VEGF (low VEGF), and 2 $\mu$ g/mL VEGF (high VEGF) in PBS were surgically inserted into the right ventricular outflow tract (RVOT) of adult rat hearts to repair transmural defects. At Week 1 and Week 4, the biomaterials were analyzed for patch thickness, cellular density and angiogenesis (i.e. vessel density and vessel diameter).

A



B



**Figure 1: Experimental details.** (A) Experimental procedure for step immobilization of VEGF and Ang1 on collagen scaffolds. (B) Experimental timeline for *in vitro* cultivation of H5V endothelial cells on collagen scaffolds.

### Results and Discussion:

The amount of VEGF immobilized on the scaffolds, as determined by VEGF ELISA, was found to be  $1.13 \pm 0.44 \text{ ng}$  for 1/2VEGF+1/2Ang1 group and  $2.33 \pm 0.36 \text{ ng}$  for VEGF and VEGF+Ang1 groups. The amount of VEGF immobilized on the scaffolds was  $1.46 \pm 0.11 \text{ ng}$  for low VEGF group and  $11.37 \pm 0.20 \text{ ng}$  for high VEGF group. The amount of Ang1 immobilized on the scaffolds, as determined by Ang1 ELISA, was found to be  $0.30 \pm 0.04 \text{ ng}$  for 1/2VEGF+1/2Ang1 group and  $0.59 \pm 0.10 \text{ ng}$  for Ang1 and VEGF+Ang1 groups.

High endothelial cell density within the scaffold is the first step in achieving vascularization in a tissue construct. *In vitro* results (**Figure 2A**) showed that collagen scaffolds with immobilized VEGF (VEGF+PBS, 1/2VEGF+1/2Ang1 and VEGF+Ang1 groups), had increased final cell number (1.6-fold, 1.8-fold and 1.7-fold respectively) compared to PBS control at Day 3 (one-way ANOVA,  $P = 0.0213$ ). This is consistent with the cell proliferative

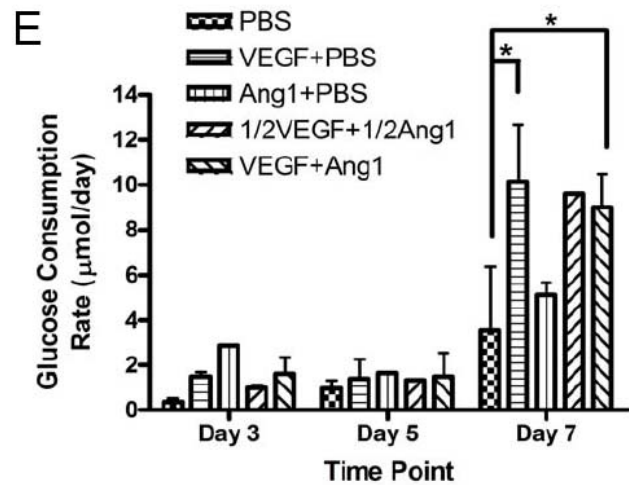
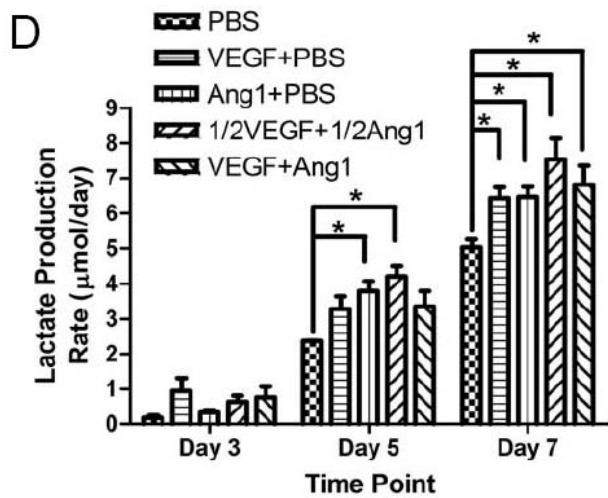
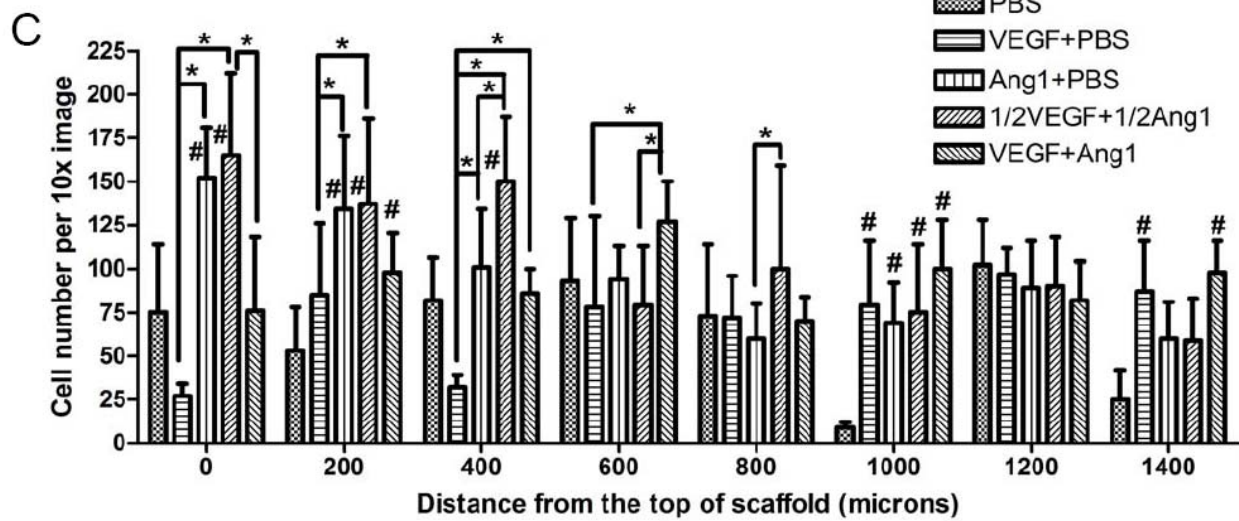
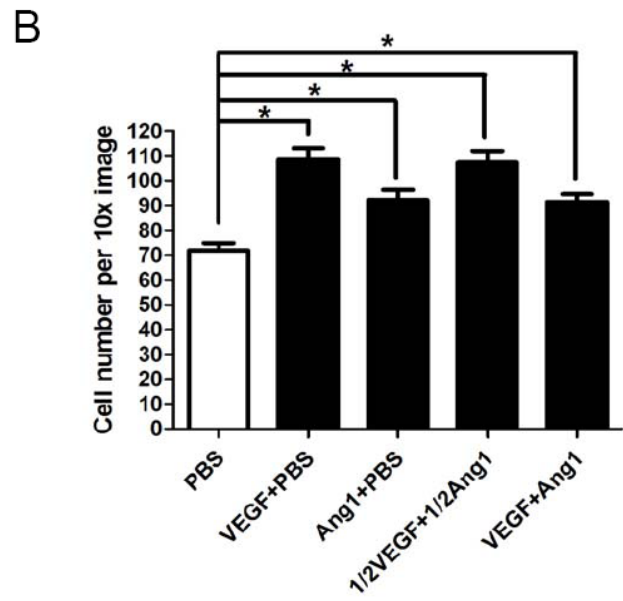
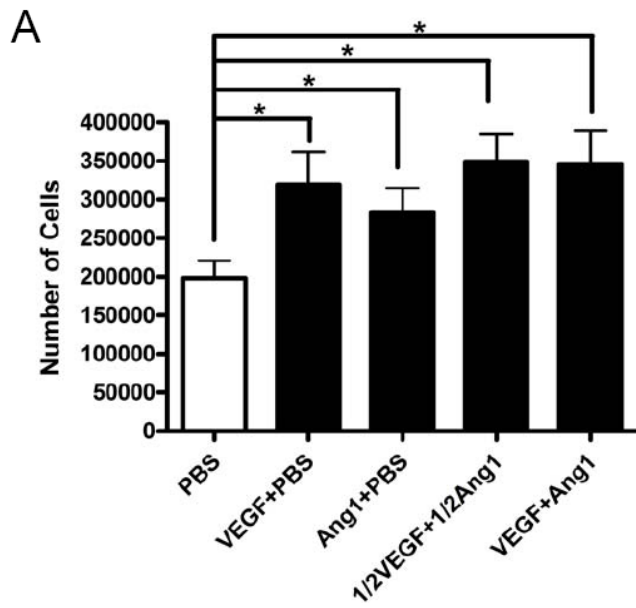
effect of VEGF. Although Ang1+PBS group had a 1.4-fold higher cell number than PBS control, it did not show statistically significant difference in final cell number compared to PBS control. This is due to the fact that Ang1 is responsible for endothelial cell survival rather than cell proliferation.

Scaffolds were also cryosectioned and stained with DAPI to count number of cell nuclei at various heights (**Figure 2B, 2C**). At Day 3, groups with immobilized VEGF and/or Ang1 all showed higher cell density (**Figure 2C**) than PBS control (two-way ANOVA,  $P < 0.0001$ ). Interestingly, at 1400 $\mu$ m from the top of the scaffold, only VEGF+PBS and VEGF+Ang1 groups (and not Ang1+PBS and 1/2VEGF+1/2Ang1 groups) showed significantly higher cell density than PBS control (**Figure 2C**), thus indicating a dependency of cell infiltration on immobilized VEGF concentration.

Lactate production rate (**Figure 2D**) and glucose consumption rate (**Figure 2E**) both increased from Day 3 to Day 7 for all experimental groups *in vitro*. More importantly, VEGF+PBS, Ang1+PBS, 1/2VEGF+1/2Ang1 and VEGF+Ang1 groups all showed higher lactate production rates (**Figure 2D**) compared to PBS control at Day 7 ( $P < 0.0001$  two-way ANOVA;  $P < 0.05$ ,  $P < 0.05$ ,  $P < 0.001$  and  $P < 0.01$  respectively Bonferroni post-tests). Ang1+PBS and 1/2VEGF+1/2Ang1 groups also had higher lactate production rates compared to PBS control at Day 5 (Bonferroni post-tests,  $P < 0.05$  for Ang1+PBS,  $P < 0.01$  for 1/2VEGF+1/2Ang1). VEGF+PBS, Ang1+PBS, 1/2VEGF+1/2Ang1 and VEGF+Ang1 groups all showed higher glucose consumption rates (**Figure 2E**) compared to PBS control at Day 7 (2.8-fold, 1.4-fold, 2.7-fold and 2.5-fold respectively). Specifically, VEGF+PBS and VEGF+Ang1 groups showed significantly higher glucose consumption rate than PBS control at Day 7 (two-way ANOVA Bonferroni post-tests,  $P < 0.05$ ).

For preliminary *in vivo* studies (data not shown), sponges were immobilized with low dose VEGF (500ng/mL VEGF) and high dose VEGF (2 $\mu$ g/mL VEGF), and used in the adult rat RVOT. It was found that the patch thickness was significantly increased with high dose VEGF compared to both PBS control and low dose VEGF at Week 1. The patch thickness was also significantly increased with low dose VEGF and high dose VEGF compared to PBS control at Week 4. The increase in patch thickness was not due to increased cellular density, since there was no statistically significant difference in cell density amongst groups at both Week 1 and Week 4. Rather, trichrome stained images and corresponding image analysis showed that blood vessel density was increased in high dose VEGF patches compared to PBS control at Week 1 and compared to both PBS control and low dose VEGF patches at Week 4.

The modification of cardiac patches with immobilized VEGF seems to increase blood vessel density in the biomaterial over time, thus increasing the thickness of the implanted VEGF patch. This is consistent with the angiogenic effect of VEGF. However, the vessel diameters were not significantly different between high VEGF and control sponges, suggesting a need for an additional growth factor such as Ang1 for vessel stabilization. Future work includes an *in vivo* study on the effect of co-immobilized VEGF and Ang1.



**Figure 2: Comparison of individually immobilized and co-immobilized growth factors *in vitro*.** (A) XTT assay indicating final cell numbers in collagen scaffolds at Day 3. (B) Averaged cell density at Day 3. (C) DAPI staining indicating cell density at various heights. (D) Lactate production rate. (E) Glucose consumption rate. \* denotes statistically significant difference, # denotes statistically significant difference compared to PBS control ( $P < 0.05$ ; one way ANOVA with post-hoc Tukey test for cell number and averaged cell density; two-way ANOVA with Bonferroni post tests for cell density, lactate production rate, and glucose consumption rates)

### Conclusions:

The covalent immobilization of combined growth factors VEGF and Ang1 in collagen sponges leads to endothelial cell proliferation and survival, and increased cell metabolism *in vitro*. More importantly, angiogenic effect was evident when collagen scaffolds with immobilized VEGF were used in preliminary *in vivo* studies to repair the right ventricular outflow tract of adult rat hearts. Further studies will be performed to investigate the effect of combined immobilized growth factors on angiogenesis *in vivo*.

### Acknowledgments:

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