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Thermoresponsive hydrogel as a delivery scaffold for transfected rat MSCs

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Abstract

The concept of stem cells as a therapeutic agent has been gaining momentum. A common mode of administration of these cells is by direct injection into the target tissue. This can result in many of the cells being lost due to reflux from the injection site leading to a local loss of implanted cells. PoligoGel is a non-toxic hydrogel with an LCST near body temperature. It is also shown to be non-toxic to multiple cell types, and in the case of rat mesenchymal stem cells does not alter their differentiative capacity, either by inducing differentiation, or limiting the potential for subsequent differentiation after removal from the gel. Embedding cells in PoligoGel also does not interfere with the cells ability to delivery therapeutic growth factors post transfection with plasmid DNA. Here a thermoresponsive hydrogel, PoligoGel, is shown to have potential to act as a scaffold for the retention of cells at an injection site, mitigating migration or washing of the cells away from the target site after implantation.

Keywords

Thermoresponsive hydrogel; MSC; transfection; cell delivery

INTRODUCTION

Cell based treatment for disease is growing in scope and effectiveness. Uses currently range from neural therapies[1], to heart treatments [2], and to cartilage [3]. This has increased the need to develop delivery strategies that are able to maximize the potential of these cellular treatments. This is especially true in the case of autografts, where limited numbers of cells can be isolated and a fast turn around time is often key [4]. While the use of cells suspended in saline is commonly demonstrated in studies, it is not perfect; only a limited number of cells may engraft at the implant site. One study found only 10% of injected cells were retained 30 minutes after a myocardial injection [5]. The authors concluded by commenting on the need of furthering the development of strategies to retain the cells where needed.

Bone marrow derived MSCs have been known for over a decade to have the potential for differentiating into multiple cell types. [6] This cell type can be obtained from the bone marrow by aspiration, resulting in large number of collected cells which do not require harsh enzymatic treatment to isolate from the host tissue, or intensive isolation procedures. In many instances all that is required is simply taking the adherent fraction of mononuclear cells; this does result in some heterogeneity in the population however.[7]

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Scaffolding systems seeded with cells is one method for limiting the departure of implanted cells from the target area. This has been accomplished by preformed scaffolds [8], or injectable *in situ* forming scaffolds which may be stiff[9] or more often a hydrogel.[10, 11] Cell sheets have also been used with success[12].

Hydrogels are ideal candidates for soft tissue as they are supple, and generally perform well from a biocompatibility perspective. Hydrogels can also be tailored by altering the crosslink density, the pore size, degradability, stiffness, and other parameters in order to achieve the requisite properties [13]. Hydrogels have long been used as a biomaterial for local deliver of small molecules including examples from the late 1970's [14]. More recently *in situ* gelling hydrogels have been used to deliver various molecules such as steroids, proteins, and chemotherapy agents [10,15–17]. Materials undergoing gelation *in situ* are especially useful due to the ease of delivery, a simple injection. Hydrogels which gel via chemical cross linking have potential drawbacks; including reactivity of active groups with host tissues, leachable small molecules, monomer or initiator, limited working time for injection prior to gelation, or the need for additional equipment, such as a uv source.

In comparison stimuli sensitive, and specifically thermoresponsive, hydrogels do not have these drawbacks. However, they can be significantly more difficult to customize without skewing the hydrophilic/hydrophobic balance and altering the LCST. The lack of covalent crosslinking also tends to yield less mechanically stable materials. Temperature and pH change can be used to initiate a phase change in these polymers leading to gelation, independently, or used in conjunction with success [15, 18]. Temperature sensitive polymers have been widely studied [19,20]. Some of these polymers, such as pluronic F127, have successfully been used for drug delivery, but have met with difficulty when used for cell encapsulation where the amphiphilic nature of the polymers can lead to membrane destabilization resulting in cell death [21]. PoligoGel is a novel degradable thermosensitive hydrogel manufactured by SamYang Corp. of Korea covered by United States Patent number 7,655,735. It is based on multiple alternating blocks of PEG and PPO or PBO joined by degradable dicarboxilic linkages and has an LCST near physiological temperatures. It is non-toxic to multiple cell lines including rMSCs, human umbilical vein endothelial cells (HUVECs), and mouse colon carcinoma (CT-26), and in the case of rMSCs does not influence their pluripotency. Here it is shown to be a suitable material for the delivery of bone marrow derived rat mesenchymal stem cells (rMSCs). When used with transfected MSCs, excreted growth factors are able to diffuse from the gel and elicit a growth response in HUVECs, allowing for a wide range of potential treatments.

Experimental

Cell culture—rMSCs were obtained from Tulane University's Center for Gene Therapy and expanded as recommended. In brief, cells were grown in Minimum Essential Media alpha (Invitrogen, Catalog number 12561-056), supplemented with 20% FBS (biowest, Catalog number S01520) and 1× Penicillin/Streptomyosin (Invitrogen, Catalog number 15140-122). Media was exchanged every 3–4 days. Cells for expansion were plated at approximately 100 cells/cm^2. Cells are passaged at a confluency of 70–90%. To lift the cells they are washed in PBS and incubated in TrypLE™ Express (Invitrogen, Catalog number 12605-010) for five minutes, or until detached from the plate. CT-26 cells were grown in RPMI-1640, 10% FBS and 1× Penicillin/Streptomyosin, but other wise cultured the same as the MSCs. HUVEC cells were grown in EGM-2 BulletKit media (Lonza CC-3162) used as supplied. Cells were passaged at 90% confluency, and media was refreshed every 2–3 days. Primary cells were used at passage numbers 10 or less.

MTT assays were performed by adding MTT to fresh growth media to a final concentration of 0.2mg/mL. Cells were incubated for 3–4 hours at 37°C 5% CO2 to allow the formation of

formazan crystals. The media was then removed, and the cells were washed with PBS. The formazan crystals were then dissolved with DMSO and the absorbance was read on a plate reader (BioRad Model 680) at 570nm.

Trypan Blue 0.4% (Sigma, T-8154) was added to cells in suspension at a 1:1 ratio. The mixture was allowed to incubate at room temperature for 5 minutes after which live and dead cells were counted on a hemocytometer.

Cell viability by alamarBlue was accomplished according to the manufactures instructions. Briefly, cells were plated in white clear bottom 96-well plates. At the required time points 10× alamarBlue was added to the culture media and allowed to incubate for between 4 hours and overnight, depending on the cell concentration. Plates were then read on a PerkinElmer LS 55 Luminescence Spectrometer.

Fibrin gels were prepared in the following manner. Fibrin powder (Sigma, F2629-5g) was reconstituted in PBS by adding the fibrin to the top of the PBS in a 50ml tube with gentle rocking and allowed to dissolve. Fibrin concentrations were calculated to be near 10mg/ml using uv/vis. Thrombin (Sigma, T4648) was reconstituted in PBS at 100 units/ml and stored at −20°C until used. Gels were made in 96 well plates by adding 20 ul thrombin to 30 uL fibrin solution.

Differentiation—Cells were grown to confluency in 6-well plates prior to adding the appropriate induction media. For osteogenic differentiation it included complete media supplemented with 10mM β-glycerol phosphate, 50 ug/ml ascorbic acid, and 100nM dexamethazone; media was refreshed every 3–4 days. Adipogenic differentiation media was used as reported by Winer et al [22]. Induction media consisted of growth media supplemented with 1 μM dexamethaxone, 200μM indomethacin, 10 μg/mL insulin, and 0.5mM 3-isobutyl-1-methylxanthine, which was placed on the cells for 3 days, followed by maintenance media (complete media with 10μg/ml insulin) for one day. This cycle was repeated twice, for a total of 8 days. All of the supplements used were obtained from Sigma, and were cell culture tested.

PoligoGel—PoligoGel (SamYang Corp.) was obtained in a lyophilized cake. To hydrate the polymer it was mixed with unsupplemented growth media or PBS and stirred overnight at 4°C to make a 10% w/v solution. For suspending cells in the gel, a10% PoligoGel solution was mixed at 4°C 1:1 with cells suspended in complete media to give a 5% polymer solution and the desired concentration of cells. The 5% solution was then placed in a cell culture incubator at 37°C. 5% final solutions were used unless otherwise noted. Properties of 5% gels are mechanically sufficient for cell delivery without the added viscosity and associated difficulty of working with higher fraction gels.

SEM Data—Samples were prepared by flash freezing in liquid nitrogen, followed by lyophilization. Samples were then imaged on a FEI Quanta 600 FEG.

RNA isolation and PCR: RNA was isolated from rMSCs using TRIzol reagent (Invitrogen, 15596-018). cDNA was created with SuperScript III First-Strand (Invitrogen, 18080-051) using oligo DT primers following the recommended protocol. PCR for amplification of the genes of interested was performed using either Kapa2G Fast PCR Kit or the Hot Start kit (KapaBiosystems). Primers used for amplification are listed in Table 1.

Transfection—Cells at about 50% confluency were transfected in serum free media and allowed to incubate for 4 hours; after which they were placed back in standard growth media

or gel mixed with growth media. Cells were transfected with branched Poly(ethyleneimine) at an N:P ratio of 10:1.

Results

Viability of cell types tested was not affected by the presence of PoligoGel at any of the concentrations tested. PoligoGel was tested on rMSCs, CT-26 (mouse colon cancer) cells, and Human Umbilical Vein Endothelial Cells (HUVECs). In all cases the same trend was observed, no toxicity at non-gelling concentrations of 0.1%, 0.5%, and 1% to a 5% or 10% gel. In each case there was no statistical difference by MTT assay at non-gelling concentrations. At 5% or higher solutions the cells under the gel did not proliferate, but remained viable as measured by Trypan blue. When confluent cells that were no longer actively dividing were used there was no significant difference between the control and experimental groups as shown in Figure 1. Groups were normalized to samples without the use of PoligoGel.

rMSCs suspended in PoligoGel, also do not suffer any significant cell death. For these studies, cells were tripsonized, pelletted, resuspended in growth media and placed on ice to lower the temperature. The cooled cell suspension was then mixed with PoligoGel, also cooled on ice, to achieve final concentrations of 100k, 200k, 500k and 1 million cells per milliliter and polymer concentrations of 5%. 500uL of the gel/cell mixtures were then placed in a 24 well plate. After 48 hours cells were removed from the gel by cooling the gel and adding sufficient media or PBS to lower the total concentration of polymer to prevent a gel from reforming. The mixture was then removed from the well and centrifuged at 400 rcf for 5 minutes to pellet the cells, which were resuspended in growth media and analyzed with trypan blue. The viabilities, ranged from 95% at 100k per ml to 85% at 1 million per ml.

Cells embedded in PoligoGel show a spherical morphology with no visible protrusion into the gel when viewed by light microscopy; not the typical spindle shape seen when cultured on substrates such as fibrin with sites for cell binding. They also do not appear to proliferate based on the observation that cells remain suspended and are typically seen independent of other cells, not forming clusters as would be expected for dividing cells unable to migrate through the substrate. Scanning electron microscopy confirms this, with cells appearing to be encased in the polymer in stark contrast to cells grown in or on a fibrin gel where cells can be seen to attach and spread, resulting in a spindle shape as seen in Figure 2.

When culturing stem cells others have observed that the substrate properties influence cell fate along different differentiative pathways.[22] Substrate properties are also important for other cell types as well. [23,24] MSCs growing in PoligoGel are exposed to a soft three dimensional scaffold, undergo a halt in proliferation, and an induced morphological change. It was possible that cellular capacity to differentiate was effected. To ensure that PoligoGel does not push rMSCs to differentiated fates, cells were cultured for 1 week suspended in the gel at about 100k cells/ml before being removed. Cells were then placed in differentiation media, or had the RNA immediately collected for PCR analysis following removal from the gel. Cells induced to an adipogenic fate were confirmed to develop into adipocytes by both the presence of lipid droplets in the cells(data not shown), as well as expression of genes indicative of an adipocyte phenotype. Control cells removed from the gel not placed in adipogenic induction media did not express the genetic markers for adipocytes; either immediately upon removal or at the time the induced cells were analyzed. While it would be unlikely for the soft gel substrate to induce differentiation of the cells to a hard tissue, the retained capacity to do so does allow for the confirmation of the cells retained pluripotency. When treated with osteogenic media cells removed from PoligoGel changed from a spindle shaped morphology to a more cuboidal shape. Cells were also harvested for RNA collection

at the end of the incubation period for PCR analysis. Cells were positive for both osteogenic markers.

Peroxisome proliferator activated receptor γ (PPAR-γ)and Adipocyte P2 (aP2) were measured for adipogenic differentiation; for bone Osteoblastic specific factor-2 also known as Runx2(OSF-2) and Osteocalcin (OCN). For each pair the first gene is an early marker and the second gene is a later marker for differentiation along that path. The early markers may be constitutively expressed at low levels. Cells removed from PoligoGel did not exhibit either of the late differentiation markers, but where was some up regulation in the expression of PPAR-γ in the cells removed from the gel. Differentiated cells did express both RNAs with or without incubation in the gel. PCR results looking for the different marker genes are shown in Figure 3.

The ability of transfected rMSCs to elicit a growth response was tested on HUVECs. As previously mentioned, cells grown under gelled PoligoGel did not proliferate. To avoid this effect, HUVECs were plated in 96 well plates, and 50 ul of a fibrin gel was placed on top of the cells. MSC growth media was added to the wells after the fibrin had form a gel, and allowed to sit overnight. The following day the excess media was removed by inverting the plate over sterile gauze, allowing the media to drain by gravity. Transfected MSCs were mixed in PoligoGel four hours post transfection, and 200 uL of the gel were added to each well. The cell concentration was approximately 100–200k cells/mL. After 48 hours for cell growth the plates were placed at 4 degrees C for 5 minutes and had 200mL cold PBS added. The MSCs, and excess PoligoGel were then removed by inverting the culture plate over sterile gauze. 200 uL of growth media were then added, above the fibrin gel and HUVEC cells. Alamar blue was then used to assay the number of cells in each well. As seen in Figure 4, all groups out performed the HUVEC only control, and all growth factor transfected groups out performed the addition of unaltered stem cells.

The persistence of the transfection was observed with PCR is shown in Figure 5. All transfections showed elevated mRNA levels of gene expression beginning at 4 hours and continuing to three days, with higher levels observed for FGF and VEGF at 7 days as well. Also of note is that the initial FGF transfection seems to have promoted an increase in the transcription of VEGF, and transfection with any of the three growth factors, but not a luciferase control, increased FGF mRNA levels. VEGF transfected cells continued to show increased FGF mRNA at three days out as well. By day seven, the expression levels had dropped back down near the levels of the control cells.

Discussion

Cell treatments will never reach their full potential if the cells fail to persist at the intended target. Cells are lost to washout from the injected site, migration, and cell death. Here it has been demonstrated that the use of a thermoresponsive hydrogel has the potential to be used for the *in vivo* delivery of cells. The use of the gel can reduce the loss of cells from the implant site due washout and migration by forming a hydrogel scaffold that will hold cells near the injection site where they will be able to affect the desired tissues; both directly as the gel degrades and cells are able to escape, and indirectly by releasing growth factors both constitutively or by means of transfection. PoligoGel can be used to potentially reduce the number of cells required for injection by keeping a greater proportion of the cells nearby. While MSCs derived from bone marrow are relatively plentiful and easy to obtain; this benefit is should not be over looked, especially for allografts of cells such as cardiomyoblast, where yields are lower and more difficult to obtain. [25]

PoligoGel was also shown to be non-toxic at low concentrations to multiple cell types although proliferation was adversely affected when a gel was formed above the cells.

Mesenchymal stem cells suspended in the gel suffered no significant adverse consequences at cell concentration up to 1 million cells per ml. Cell viability remained high, above 90%, and dropped to 84% at the higher concentration. This cell death is not assumed to be due to PoligoGel or the encapsulation process, but rather due to lack of diffusion of oxygen and metabolites for cells farther from the surface resulting in necrosis but this was not tested. Cells also do not appear to proliferate in the gel, based on the observation that cells remain suspended and are typically seen independent of other cells, not forming clusters as would be expected for dividing cells unable to migrate through the substrate. Studies using direct cell transplantations have used far greater numbers of cells for the injection, typically in the tens to hundreds of millions in humans[26]. This would require far greater cell loading than would be practical with this system, but with the cells fixed in position it is possible to have equivalent if not greater numbers of cells near the requisite site.

Maintaining viable cells is only part of the difficulty in using stem cells. The cells must retain their differentiative capacity in order to achieve the full therapeutic potential. PoligoGel does not have any observed effect on mesenchymal stem cells ability to differentiate after 1 week of being exposed to the gel; either by pushing the cells to a differentiated fate, or limiting their ability to become terminally differentiated cell types. This allows for local cellular signals at the implant site to push the stem cells to differentiate into suitable cell types for wound healing; whether that is endothelial cell or smooth muscle cell for revascularization, or cardiomyocytes for replacing cells which may have died during the ischemic event.

Transfected cells can improve the outcome by delivering growth factors that can recruit additional cells to the tissuse and also promote survival of the native cells. The use of PoligoGel does not impair the effectiveness of the transfection. Transfected cells in suspended in the gel were able to elicit a growth response from HUVECs; a luciferase assay further confirmed no statistical difference in protein production between cells in the gel and those on standard surfaces (data not shown). Looking at mRNA expression demonstrated a typical expression profile with a peak at 3 days, and finished near 7 days.

Conclusions

With the growing number of cell therapies being tested and developed it is important to ensure that the cells are being used to the greatest advantage. The thermoreversible polymer PoligoGel has been shown to have the potential to be used for the delivery of stem cells. It does not influence stem cell differentiation and is non-toxic. Transfected cells embedded in the gel have been shown to be able to produce therapeutic proteins which can be delivered from the gel eliciting a growth response in other cell types.

Acknowledgments

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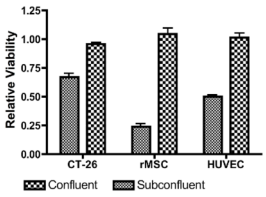

Figure 1.

While the gel does not induce cell death, as confirmed by trypan blue staining, it does limit proliferation. When placed above confluent cells there is no change against confluent controls. Variations in the subconfluent values are based on differences in the initial number of cells present.

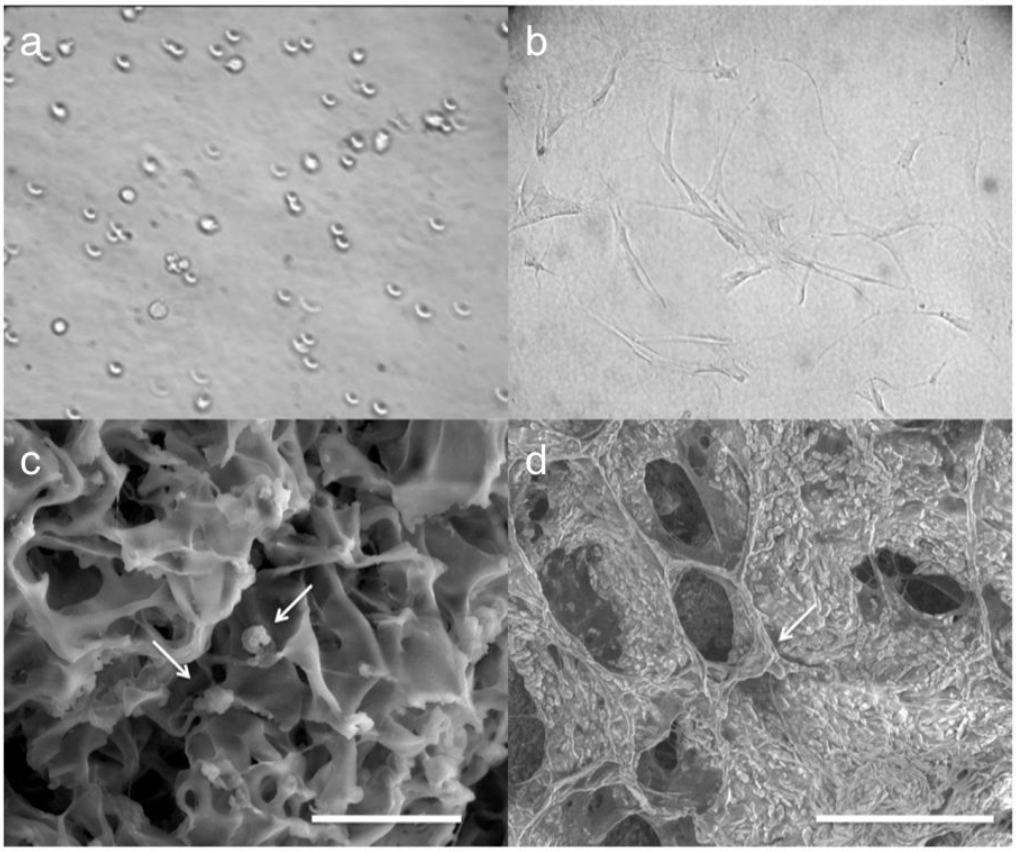

Figure 2.
Images of rMSCs on PoligoGel and Fibrin Gels. a) image taken with a light microscope with cells in PoligoGel 5%, b) light microscope image of rMSCs growing on a fibrin gel, c) SEM image of rMSCs embedded in PoligoGel, d) fibrin gel with rMSCs. While the fibrin promotes cell attachment and spreading, no evidence is seen of such activities in the PoligoGel samples. Scale bars are 50 microns in the SEM images.

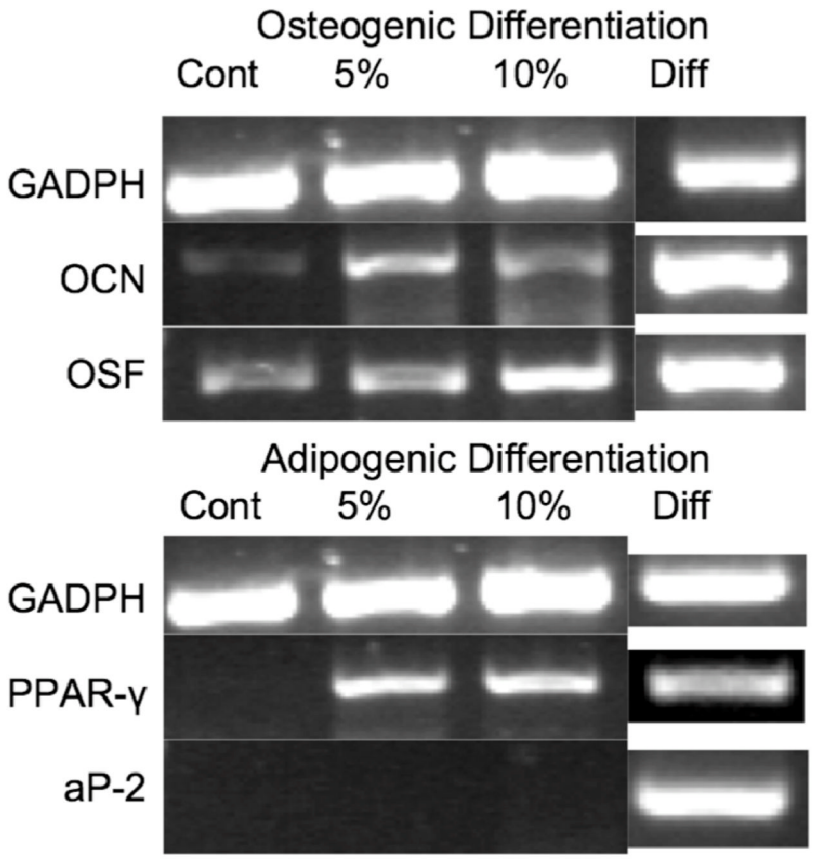

Figure 3.

MSC differentiation did not occur as a result of exposure to PoligoGel at either a 5% or 10% concentration although they did express low levels of some factors compared to control groups. Cont – control cells grown on standard tissue culture surfaces; 5% - Cells grown in 5% PoligoGel; 10% - Cells grown in 10% PoligoGel; Diff –Control cells exposed to differentiation media without prior exposure to PoligoGel. Cells removed from PoligoGel retained the ability to differentiate as evidenced by production of both markers.

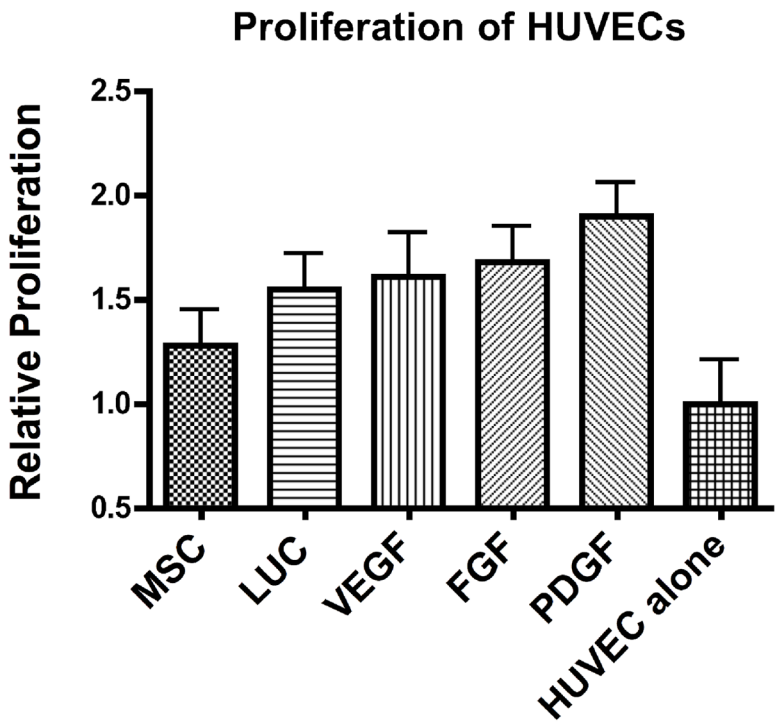

Figure 4.

All transfected groups were significantly improved over HUVEC only group $p < 0.05$. PDGF-C was significantly different from all non-growth factor sets at $p < 0.05$, not the difference was not significant between the growth factor transfected groups. Data is the average of 6 wells, error bars are the standard deviation. Data was analyzed by ANOVA with Bonferroni post tests to test differences between groups.

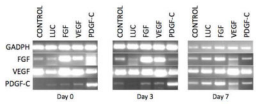

Figure 5.
PCR data showing the expression of various growth factors both constitutively and as a result of transfection. Rows are genes of interest, and columns show the gene the cells were transfected with if applicable. Elevated mRNA levels were seen for both FGF and VEGF out to 7 days. With both of them positively influencing the expression of the other.

Table 1

List of primers used in PCR for amplification of target genes.

Gene	Forward Primer	Reverse Primer
aP2	AATTCGGCACGACTCCTTGAAAGC	TGGTCGACTTTCCATCCCACTTCT
PPAR-γ	TCTCCAGCATTTCTGCTCCACACT	ATACAAATGCTTTGCCAGGGCTCG
Runx2	CAAGTGCGGTGCAAACTTTCTCCA	TGTTTGACGCCATAGTCCCTCCTT
Osteocalsin	AATGCCACTGCGTATTGGTTGACG	TGGCGGTGAGATAATGGATGTGGT
GADPH	GACCCCTTCATTGACCTCAACTAC	AGATGATGACCCTTTTGGCTCC
VEGF	GCCAGCACATAGGAGAGATGAG	GCTTGTCACATCTGCAAGTACG
FGF	CAAGCAGAAGAGAGAGGAGTTGTGTC	TCAGCTCTTAGCAGACATTGGAAG
PDGF-C	TCCTGCTGACATCTGCCCTG	CAGGAGACAACCTGGCCAG