Calcium-Alginate Hydrogel-Encapsulated Fibroblasts Provide Sustained Release of Vascular Endothelial Growth Factor

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Vascularization of engineered or damaged tissues is essential to maintain cell viability and proper tissue function. Revascularization of the left ventricle (LV) of the heart after myocardial infarction is particularly important, since hypoxia can give rise to chronic heart failure due to inappropriate remodeling of the LV after death of cardiomyocytes (CMs). Fibroblasts can express vascular endothelial growth factor (VEGF), which plays a major role in angiogenesis and also acts as a chemoattractant and survival factor for CMs and cardiac progenitors. In this *in vitro* model study, mouse NIH 3T3 fibroblasts encapsulated in 2% w/v Ca-alginate were shown to remain viable for 150 days. Semiquantitative reverse transcription–polymerase chain reaction and immunohistochemistry demonstrated that over 21 days of encapsulation, fibroblasts continued to express VEGF, while enzyme-linked immunosorbent assay showed that there was sustained release of VEGF from the Ca-alginate during this period. The scaffold degraded gradually over the 21 days, without reduction in volume. Cells released from the Ca-alginate at 7 and 21 days as a result of scaffold degradation were shown to retain viability, to adhere to fibronectin in a normal manner, and continue to express VEGF, demonstrating their potential to further contribute to maintenance of cardiac function after scaffold degradation. This model *in vitro* study therefore demonstrates that fibroblasts encapsulated in Ca-alginate provide sustained release of VEGF.

Introduction

A MAJOR PROBLEM after myocardial infarction (MI) is rapid death of cardiomyocytes (CMs) followed by inappropriate left ventricle (LV) remodeling, leading to congestive heart failure in response to the hypoxic environment.^{1–3} Current treatments to prevent progression of inappropriate LV remodeling include the use of artificial prostheses⁴ and therapeutic molecules such as angiotensin-converting enzyme inhibitors and/or beta-blockers.^{5,6} The need for other treatments, however, is clear, since these treatments do not provide regeneration and functional recovery of the ventricle.⁴ Furthermore, many ventricles continue to enlarge,^{7,8} with MI still often progressing to chronic heart disease³ causing morbidity and mortality rates to remain high.⁹

At present, there is a great interest in the development of cell-based therapies for cardiac repair. Initially, researchers investigated whether directly injecting cells into the infarcted region could stimulate regeneration.^{4,10,11} This approach was seen to be of limited benefit, likely due to lack of cell retention and survival at the injected site.^{12,13} More recently, re-

searchers have looked at delivering cells to the infarcted region with a biomaterial scaffold. The scaffold can retain the cells at the desired location, thus preventing physical loss and protecting them from the harsh hemodynamic environment of the heart.^{4,14,15} Scaffolds also provide other beneficial effects, for example, mechanical stabilization and preservation of elasticity in the infarcted area of the heart, preventing fibrotic tissue formation and ventricle enlargement by reducing wall stress due to scar thickening.^{16,17}

Alginate is a good candidate material for delivery of cells to the infarcted heart, because it is readily available, does not facilitate pathogen transfer, is nontoxic, nonimmunogenic, and gels in the presence of calcium to form a hydrogel with a high water content that allows good exchange of waste products and nutrients.^{1,2,18} Alginate hydrogels have been successfully used to encapsulate a variety of cell types for tissue engineering applications. In addition, Ca-alginate gels implanted into the heart have been shown to be well tolerated and not to provoke adverse responses such as thrombosis¹ or fibrosis, even when used to provide localized release of VEGF.¹⁹ Ca-alginate gels have also been

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demonstrated to provide a temporary physical support to the damaged tissue, replacing some of the roles of the extracellular matrix (ECM) while also preventing adverse remodeling and dysfunction in rat models of both recent and old MIs.²⁰ The gradual Ca-alginate gel degradation in physiological conditions by dissipation of calcium crosslinks²¹ results in water-soluble nontoxic alginate polysaccharide degradation products, which are excreted in the urine.²² Additionally, we have previously shown that fibroblasts encapsulated in Ca-alginate have a very low metabolic demand and mitotic activity,²³ and therefore are likely to survive in the harsh hypoxic environment of the infarcted LV.²

Many cells have been suggested to be beneficial in improving the structure and function of a postinfarcted heart. There has been research into the beneficial outcome of delivering CMs, since these cells are responsible for the contractile function of the heart. Since the number of proliferative CMs that can be obtained from a heart are low, other cell therapies have also been investigated.²⁴ The benefits of delivering stem cells, including embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells, which can differentiate to give rise to CMs,¹⁰ have been investigated. Limited success has, however, been realized using stem cells, and concerns remain regarding the potential cancer risk^{25,26} and adverse immune response²⁵ associated with their use. Recent research has also shown the potential benefit of delivering fibroblasts to the infarct region.^{27,28} Fibroblasts comprise about 70% of the total cells present in the heart.²⁹ They secrete the ECM and a number of growth factors, in particular vascular endothelial growth factor (VEGF),^{28,29} which has a key role in the promotion of angiogenesis,^{30,31} and acts as a mitogen, survival factor, and chemoattractant for endothelial cells (ECs).^{32–35} Since vascularization is a prerequisite for the survival and function of any transplanted or recruited cells, this feature is of great interest. VEGF is also known to play other key roles in cardiac repair.^{25,36–39} VEGF is known to be a survival factor for CMs³⁴ and maintain viability, recruit, and stimulate differentiation of resident cardiac stem cells.^{4,37–39} Human dermal fibroblasts encapsulated within a biodegradable mesh of vicryl fibres and cell-secreted ECM,⁴⁰ now known as Anginera™ (Theragen Inc.), has been shown to be a potentially effective treatment to maintain myocardial function after MI, and this product is now at the point of clinical trials. Fibroblasts in Anginera™ have been shown to remain viable when implanted into infarcted rat hearts⁴¹ and express angiogenic factors such as VEGF.^{40,41} Implantation into infarcted mouse⁴² and rat⁴¹ hearts was shown to increase vessel formation. In rats, it was shown to improve myocardial blood flow to the infarcted region and shown to improve LV remodeling and function.⁴⁰ Additionally, this product has been shown to be effective in the treatment of chronically ischemic canine⁴³ and rat²⁷ hearts, resulting from MI. The beneficial effects of the product have been attributed to the sustained localized expression of VEGF and other angiogenic factors by the fibroblasts.⁴⁰

In this study, we encapsulated fibroblasts in an alginate hydrogel and evaluated the potential effectiveness of the construct to improve cardiac remodeling after MI. The viability of encapsulated cells, their ability to provide sustained release of VEGF, and the degradation of the scaffold and potential of released fibroblasts to further contribute to

ventricular repair by continued expression of VEGF were evaluated.

Methods and Materials

Unless stated, materials were obtained from Sigma, United Kingdom.

Cell culture

NIH 3T3 fibroblasts were obtained from (European Collection of Cell Cultures). All 3T3 fibroblast cultures were maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% v/v penicillin-streptomycin, 10% v/v heat-inactivated fetal bovine serum (FBS), and 1% v/v fungizone (Gibco). The medium was changed three times weekly, and cells were cultured at 37°C in 5% CO₂.

Fibroblast encapsulation in alginate hydrogels

NIH 3T3 fibroblasts were suspended at a density of 7.5×10^5 cells/mL in 2% w/v low-viscosity sodium-alginate (20–40 centipoise (cps) for 2% w/v at 25°C; Sigma, Cat number 180947, lot 08620BJ, MW 102000–209000, M:G ratio 1.56). The alginate was gelled by adding the solution dropwise to 100 mM CaCl₂, to produce beads of 4.17 ± 0.08 mm diameter. The resulting beads were incubated in the CaCl₂ solution at 37°C for 2 h to allow complete gelation. The CaCl₂ was then removed, and the beads were washed thoroughly in the DMEM before being cultured in a supplemented culture medium (described above).

Release of fibroblasts from alginate hydrogels

Fibroblasts were released from alginate hydrogel beads at 1 and 3 weeks by immersion of the beads in a 100 mM trisodium citrate (pH 7; Sigma) for 40 min at 37°C until the beads dissolved. The solutions were then centrifuged at 1000 rpm to pellet the released cells, which were resuspended in the culture medium and plated for immunocytochemistry, VEGF Enzyme-linked immunosorbent assay (ELISA), semiquantitative reverse transcription-polymerase chain reaction (sqRT-PCR), and light-microscopy evaluation.

Coverslip preparation

Thirteen-millimeter-diameter glass coverslips (VWR International) were autoclaved then coated in poly-L-lysine (PLL) by immersion in 0.5 mg/mL PLL in ultrapure water for 20 min before removal and air-drying for 20 min. Coverslips were subsequently coated with 40 µg/mL fibronectin for 20 min at room temperature and further air-drying for 20 min.

Live/dead staining

Live/dead staining of cells encapsulated in the center of alginate beads at 150 days was performed as previously described, to show live cells in green and dead cells in red.²³

Light microscopy

The appearance of fibroblasts released from alginate hydrogels at weeks 1 and 3 were compared with control

samples (which had not been encapsulated in alginate hydrogel) using light microscopy. All fibroblasts were cultured on fibronectin-coated 13-mm coverslips (VWR) in 24-well plates (Iwaki). Released fibroblasts were plated onto the coverslips at a density of 1×10^4 cells/well, and images obtained at 7 days postplating using a microscope attached to a camera and Axiovision Rel. 4.8 software (Zeiss).

Gel degradation analysis

Five hundred microliters of samples of alginate beads were prepared and immersed in 4 mL of supplemented culture medium. The alginate hydrogel masses were assessed at various time points after *in vitro* culture. Masses were measured after blotting the alginate hydrogel beads carefully on paper towels to remove excess surface moisture and then weighed on a four-decimal-place top-pan balance (Sauter AR1014). Samples were measured in triplicate, and the mean $\pm$ SD for each data point is shown. In addition, the diameter of five beads at each time point were carefully measured using digital calipers (Fisher Scientific), and the mean $\pm$ SD values for each data point are shown.

Vascular endothelial growth factor enzyme-linked immunosorbent assay

The amount of VEGF secreted by control 3T3 fibroblast monolayers and fibroblasts encapsulated in 2% w/v alginate hydrogels was determined by VEGF ELISA (R&D Research Systems). 3T3 monolayers were seeded at a density of 4.4×10^5 /well of a six-well plate, and 1 mL of samples of alginate containing 7.5×10^5 /mL was used. Samples were cultured in 4 mL of supplemented culture medium that was changed three times weekly and left unchanged for 3 days before sampling. Medium samples were collected and stored at -20°C for up to 4 weeks before assaying, in accordance with the manufacturer's instructions. Measurements were performed in triplicate, and mean $\pm$ SD values are shown.

Semiquantitative reverse transcription-polymerase chain reaction

RNeasy minikits (Qiagen) and DNase kits (Qiagen) were used to extract mRNA. mRNA quality and quantity were evaluated and quantified using a spectrophotometer (BioPhotometer) and by electrophoresis using a 1% w/v agarose gel with ethidium bromide (Promega).

Omniscript reverse transcriptase, oligo-dT primer (0.5 μg /mL), and RNase inhibitor (40 units/ μL) from Promega were used for reverse transcription of mRNA to complementary DNA (cDNA) according to the manufacturer's instructions. cDNA was purified using Montage PCR centrifugal filters

(Millipore), and both cDNA and mRNA quality and quantity were evaluated using a spectrophotometer (BioPhotometer). DNA from three repeats was evaluated by sqRT-PCR. A master cycler thermal cycler and REDTaq® ReadyMix™ PCR reaction mixture were used to amplify the cDNA according to the manufacturer's instructions. The PCR program was as follows: 94°C for 5 min, denature the template at 94°C for 20 s, anneal primers at 60°C for 20 s, extension at 72°C for 20 s, and 72°C for 10 min. Thirty cycles were performed, and then samples were held at 4°C . All primers were designed using Primer 3 and obtained from Invitrogen. The primers used and expected band sizes are summarized in Table 1. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was normalized between samples in each set, so that the relative expression of VEGF could be established. Polymerase chain reaction (PCR) products were run on a 1.5% w/v agarose electrophoresis gel with ethidium bromide, and then images captured after UV illumination using GeneSnap software (Syngene). Quantification of VEGF band intensity, relative to GAPDH intensity, was compared between samples using GeneTools (Syngene).

Immunocytochemistry and immunohistochemistry

Samples of cells encapsulated in alginate hydrogel were collected at 1 and 3 weeks and prepared for immunostaining as previously described.²³ Cultured cells were fixed in 4% paraformaldehyde in PBS for 10 min, and then washed thoroughly and stored at -80°C . Before staining, cells were brought to room temperature.

Sections and cultured cells were stained as follows: Cells were permeabilized by immersion in 0.5% Triton X-100 (Sigma) for 10 min, then washed 2×5 min in PBS (cells) or Tris-buffered saline (TBS; alginate sections), and blocked for 30 min in 10% v/v FBS (Sigma)/PBS or TBS. The primary antibodies diluted in 2% v/v FBS/PBS or TBS were then applied to the cells and incubated overnight in a humidity chamber at 4°C . Samples were then washed 3×5 min in PBS or TBS before the secondary antibodies diluted in 2% v/v FCS/PBS, or TBS were applied and incubated in a humidity chamber for 1.5 h at room temperature. The samples were then washed 3×5 min in PBS or TBS then mounted with Vectashield hardset mounting medium with DAPI (Vector laboratories). Negative controls were performed using sections and cells with no primary antibody to see that the staining was specific. The mountant was allowed to dry overnight at 4°C , and then samples were captured using an Axio Imager fluorescent microscope (Zeiss) connected to a camera using Axiovision Rel 4.8 (Zeiss) software. All staining was performed in triplicate to ensure that presented images were representative. Exposure times were kept the same for each antibody.

TABLE 1. PRIMERS USED FOR SEMIQUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION EXPERIMENTS

Primer	Gene	Primers (forward (F) and reverse (R))	Product length (bps)
mVEGF	Mouse vascular endothelial growth factor	F-CTGCTCTGGGTCCACTGG R-CACCGGGTTGGCTTGTCACAT	431, 563, 635
mGAPDH	Mouse glyceraldehydes 3-phosphate dehydrogenase	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	450

VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Primary antibodies used were anti-VEGF (Santa Cruz; 1/200) and anti-vinculin (1/100). Green Alexa Fluor secondary antibodies (Invitrogen) were used (1/800). Rhodamine-conjugated phalloidin (Invitrogen) was added with the secondary antibody for vinculin to stain actin filaments (1/50).

Results

Mouse NIH 3T3 fibroblasts were encapsulated in 2% w/v Ca-alginate, which was evaluated *in vitro* to evaluate for the potential of fibroblasts encapsulated in Ca-alginate to stimulate ventricular repair after MI. The viability of encapsulated fibroblasts after only 3 days was compared with viability at 150 days postencapsulation using live/dead staining (Fig. 1). Almost all cells stained green rather than red in alginate hydrogels at both time points, indicating the sustained viability of alginate encapsulated cells. Furthermore, the staining indicated that a large number of cells remained immobilized within the hydrogel, even after 150 days.

Fibroblasts were shown to express VEGF before encapsulation by immunocytochemistry (ICC) (Fig. 2B). This staining was seen to be specific since no false-positive staining was detectable in the negative control (Fig. 2A). We evaluated whether fibroblasts encapsulated in Ca-alginate were able maintain to expression of VEGF by immunohistochemistry (IHC) (Fig. 3). The staining indicated that fibroblasts encapsulated in 2% w/v alginate hydrogels at both 1 and 3 weeks expressed VEGF. The level of VEGF expression appeared comparable between encapsulated fibroblasts at 1 and 3 weeks, and also seemed to be comparable, or perhaps even greater than before encapsulation (Fig. 2B). All fibroblasts in each of the encapsulated samples (Fig. 3) were seen to express VEGF, as was the case in the control sample (Fig. 2B).

The amount of VEGF released into the culture medium over a period of 21 days was measured by VEGF ELISA (Table 2). The VEGF ELISA demonstrated that fibroblasts encapsulated in alginate hydrogel were able to provide a sustained and relatively constant release of VEGF, throughout the course of the study of 1.3–1.6 ng VEGF/million fibroblasts in 3 days. The level of expression was shown not to vary significantly ($p > 0.05$) over the course of the study.

Interestingly, the secretion of VEGF protein from encapsulated fibroblasts was significantly lower ($p < 0.05$) than from fibroblast monolayers, which expressed 5.5 ± 0.6 ng VEGF/million cells in 3 days.

Over time, the cell-encapsulating alginate hydrogels degraded by release of cross-linking calcium ions into the culture medium. This degradation was characterized by an increase in the diameter of cell-encapsulating alginate hydrogel beads (Fig. 4A) and a concurrent increase in the hydrated mass of the alginate hydrogel samples (Fig. 4B).

As the alginate hydrogel degraded, fibroblasts were released and adhered to the surface of the culture vessels. Fibroblasts released from the Ca-alginate by trisodium citrate degradation of the hydrogel after 1- and 3-week encapsulation were then seeded onto fibronectin-coated coverslips and cultured for 7 days. To assess whether fibroblasts functioned normally after release from alginate hydrogel encapsulation, the morphology, viability, and ability of released cells to form focal adhesions and a normal cytoskeleton were evaluated (Fig. 5). The morphology of the released cells after both 1- and 3-week encapsulation was comparable with the control fibroblasts (Fig. 5A–C). The viability of released cells was comparable with control fibroblasts, as shown by live/dead staining to stain viable cells green and dead cells red (Fig. 5D–F). To further confirm normal behavior of fibroblasts released from Ca-alginate, released fibroblasts were stained with vinculin and phalloidin, and compared with controls. Staining for vinculin (green) was used to show presence of focal adhesions, since it is a key component of these structures. Focal adhesions formed around the periphery of released fibroblasts, at both 1 and 3 weeks (Fig. 5H, I), as they did before encapsulation (Fig. 5G). Rhodamine-conjugated phalloidin was used to stain the actin of cell cytoskeletons red. The rhodamine staining illustrated that normal cytoskeleton formation occurred in cells released from encapsulation at both 1 and 3 weeks (Fig. 5H, I), when compared with the control (Fig. 5G).

Since released fibroblasts appeared to have normal behavior, it was expected that these cells would also continue to express VEGF, as they did both in the control sample (Fig. 2) and during encapsulation (Fig. 3). Fibroblasts released at 1 and 3 weeks were stained for VEGF by ICC to investigate whether this VEGF expression was maintained (Fig. 6). Both

FIG. 1. Live/dead staining of fibroblasts encapsulated in alginate hydrogel at (A) 3 days and (B) 150 days post-encapsulation. Green staining indicates viable cells, whereas red staining indicates dead cells. The photomicrographs illustrate that the majority of cells in both alginate samples were viable at both 3 days and 150 days postencapsulation, and therefore alginate encapsulation sustained viability of fibroblasts. Color images available online at www.liebertpub.com/tea

FIG. 2. (A) Vascular endothelial growth factor (VEGF) immunohistochemistry (IHC) negative control (fibroblast monolayer with no primary antibody) and (B) VEGF positive control (fibroblast monolayer before encapsulation). VEGF is labeled green, and the cell nuclei are stained blue with DAPI showing that VEGF was expressed by fibroblasts before encapsulation in alginate hydrogel, and very little nonspecific staining is obtained. Color images available online at www.liebertpub.com/tea

samples of released fibroblasts expressed VEGF at a level that was comparable between samples and also with the level seen before encapsulation (Fig. 2B). As with encapsulated fibroblasts (Fig. 3) and control fibroblasts (Fig. 2B), all cells in each sample were seen to express VEGF.

Confirmation that VEGF expression was maintained by fibroblasts both during encapsulation and after release from the degraded Ca-alginate was then sought by sqRT-PCR analysis of VEGF expression (Fig. 7). Expression of VEGF by encapsulated fibroblasts and fibroblasts released after 1 and 3 weeks was compared with the expression of VEGF by control fibroblast monolayers. Evidence of substantial VEGF expression was evident in all five samples, and three bands corresponding with VEGF isoforms 121, 165, and 189 were seen. VEGF 121 expression was very low in all five samples and only apparent on very close examination of the bands. In contrast, VEGF 165 and 189 isoforms were high in all samples. VEGF expression levels fluctuated slightly between samples, which is to be expected due to the semiquantitative nature of the analysis. Overall, however, the results indicate that VEGF expression was maintained in all samples at a similar level.

Discussion

In this *in vitro* model study, we have demonstrated that mouse NIH 3T3 fibroblasts encapsulated in 2% w/v Ca-

alginate remained viable (Fig. 1) and continued to express VEGF after both 1- and 3-week encapsulation, by both IHC and sqRT-PCR (Figs. 3 and 7). The level of VEGF expression at both 1- and 3-week encapsulation was seen to be comparable with before encapsulation (Figs. 2, 7). VEGF ELISA demonstrated that VEGF was released from the fibroblast-alginate hydrogels at a sustained rate (Table. 2).

Interestingly, however, the amount of VEGF that was released into the culture medium over the 21 days was significantly lower than by nonencapsulated fibroblasts. This was possibly due to electrostatic interactions between VEGF and alginate polysaccharide, which have overall positive and negative charges, respectively, under physiological conditions.⁴⁴ Most of the VEGF, therefore, is proposed to remain within the gel rather than being released into the culture medium over the initial 21 days, which results in slow release of VEGF from alginate hydrogels.⁴⁵ Over the 150 days that fibroblasts were shown to remain viable (Fig. 1), however, the scaffold should become saturated with VEGF, and the release of VEGF should reflect the true secretion rate of encapsulated cells. Electrostatic interactions between VEGF and alginate have previously been exploited to prevent burst release of VEGF.⁴⁵ Retention of VEGF within the scaffold may facilitate better ventricular repair than total release through prolonged activation of the receptor VEGFR2 in ECs⁴⁶ and by creating a concentration gradient of VEGF in the initial 21 days to promote recruitment of ECs into the

FIG. 3. VEGF IHC for encapsulated fibroblasts at (A) 1 week and (B) 3 weeks. VEGF is labeled green, while nuclei are stained blue with DAPI. At both time points, expression of VEGF was detected at a comparable level. VEGF expression does not seem to be lower than before encapsulation (Fig 2B). Color images available online at www.liebertpub.com/tea

TABLE 2. AMOUNT OF VEGF PROTEIN SECRETED INTO THE CULTURE MEDIA BY FIBROBLASTS ENCAPSULATED IN 2% W/V ALGinate HYDROGEL AS DETERMINED BY VEGF ELISA UP TO 21 DAYS POSTENCAPSULATION

Time (days)	VEGF (ng) secreted by 1×10^6 cells over 3 days
7	1.5 ± 0.1
10	1.4 ± 0.0
14	1.4 ± 0.0
17	1.4 ± 0.1
21	1.4 ± 0.0

Mean values $\pm$ SD are shown ($n=3$). The secretion of VEGF by encapsulated fibroblasts was seen to be constant throughout the course of the study at ~ 1.4 ng/million fibroblasts in 3 days. The secretion of VEGF protein was, however, seen to be significantly ($p < 0.05$) lower from encapsulated fibroblasts than from fibroblast monolayers, which expressed 5.5 ± 0.6 ng VEGF/million cells in 3 days.

ELISA, enzyme-linked immunosorbent assay.

scaffold.⁴⁷ This should facilitate effective vascularization of the scaffold.

The maintained localized retention and gradual release of VEGF at the site of implantation should also promote survival of remaining CMs³³ and promote recruitment, survival, and cardiac differentiation of resident cardiac stem cells.^{4,37–39}

Degradation of the Ca-alginate scaffold was associated with an increase in bead diameter and increase in wet mass due to swelling of the hydrogel (Fig. 4), in agreement with the degradation seen in other Ca-alginate hydrogels.^{48,49} This is converse to the degradation of many other scaffold materials, such as collagen and other ECM-derived molecules, currently used for creation of cardiac patches, which decrease in mass and dimensions over time.⁵⁰ Swelling of the Ca-alginate hydrogel results from the release of calcium crosslinks into the culture medium,²¹ and is associated with

an increase in pore size.^{50,51} This allows increased diffusion⁵² of nutrients and waste products to occur with scaffold degradation to maintain viability of increasing cell numbers that are likely to occur due to migration of cells into the scaffold after implantation. The relatively slow degradation of the scaffold, compared with materials such as collagen, will also ensure that an area for CMs and ECs to invade is maintained over longer time period, while retaining implanted fibroblasts at the desired implantation site to generate prolonged localized VEGF release. Furthermore, it should prevent the formation of dense noncontractile fibrotic scar tissue and provide mechanical stability to the infarct region to prevent LV expansion.^{16–18}

During degradation of the alginate hydrogels, cells were released from encapsulation. We showed that released cells were able to adhere to culture substrates and maintained similar viability to nonencapsulated fibroblasts that formed focal adhesions and had normal cytoskeletal arrangement (Fig. 5). The released fibroblasts were shown to continue expressing VEGF by IHC (Fig. 6) and sqRT-PCR (Fig. 7). These findings indicated that Ca-alginate encapsulation had no adverse effect on the fibroblasts, even after their release from the hydrogel, and that as the scaffold degrades *in vivo*, the released fibroblasts should adhere within the local environment and continue to contribute to expression of VEGF to maintain myocardial function.

Future work should seek to evaluate the effectiveness of alginate-encapsulated autologous fibroblasts to promote appropriate LV remodeling after MI *in vivo*. Differences in the expression of VEGF isoforms are known between mouse and human fibroblasts, with human fibroblasts expressing predominantly VEGF¹²¹, some VEGF¹⁶⁵ and very little VEGF^{189, 53–55} in contrast to the expression of mainly VEGF¹⁶⁵ and VEGF¹⁸⁹ isoforms in mouse fibroblasts and a small amount of VEGF¹²¹, as observed in this study. It is therefore important that future *in vivo* work toward clinical applications is performed with human fibroblasts, since different VEGF isoforms demonstrate different modes of action.^{35,53} Oxygen tension will likely be low within the

FIG. 4. Change in (A) bead diameter and (B) wet mass of fibroblast-encapsulating alginate hydrogel beads over a period of 21 days. Over the 21 days, the alginate hydrogel swelled due to the release of calcium crosslinks, and this was associated with an increase in bead diameter (A) and an increase in the mass of the gel (B) due to uptake of water.

FIG. 5. Phase-contrast images of fibroblasts before encapsulation (A), released after 1-week encapsulation (B), released after 3-week encapsulation (C). Live (green)/dead (red) staining (D–F) and vinculin (green)/phalloidin (red) staining (G–I) of corresponding fibroblast samples. Images show the normal morphology (B, C), viability (E–F), and focal adhesion distribution and cytoskeletal arrangement (H, I) of 3T3 fibroblasts released from alginate hydrogel after both 1 and 3 weeks, when compared with control fibroblasts (A, D, G). Color images available online at www.liebertpub.com/tea

alginate scaffold when implanted into an infarcted heart, due to the initial lack of vasculature,⁵⁶ and this may result in increased VEGF expression through the upregulation of hypoxia-induced factor 1 α (HIF-1 α),⁵⁷ especially as fibroblast viability will likely be maintained due to the low metabolic demand of fibroblasts encapsulated in Ca-alginate²³ and the demonstrated retention of fibroblast viability when implanted into infarcted heart within an alternative scaffold.⁴¹

Conclusion

Fibroblasts encapsulated in Ca-alginate can provide sustained localized retention and release of VEGF. After scaffold degradation, released cells remain viable, adhere to fibronectin, and continued to express VEGF. Alginate-encapsulated fibroblasts may therefore be useful to maintain myocardial function after MI by induction of angiogenesis. Future work should seek to ascertain the suitability of

FIG. 6. VEGF expression by fibroblasts after release from alginate hydrogel after (A) 1-week and (B) 3-week culture. VEGF is labeled green, and cell nuclei stained blue with DAPI. This illustrates that after alginate degradation and the release of entrapped cells, cells retain the ability to express VEGF. Color images available online at www.liebertpub.com/tea

FIG. 7. Reverse transcription-polymerase chain reaction analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and VEGF expression by 3T3 fibroblasts after encapsulation in alginate hydrogel for 1 or 3 weeks, compared with before encapsulation (control). GAPDH was used for normalization of samples. VEGF expression was seen in all encapsulated and released samples at a level comparable with control. Three bands were seen for VEGF, which corresponded with the isoforms VEGF 121, 165, and 189.

alginate-encapsulated human fibroblasts to prevent LV deterioration and to promote appropriate LV remodeling after MI *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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