

Encapsulation of factor IX–engineered mesenchymal stem cells in fibrinogen–alginate microcapsules enhances their viability and transgene secretion

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Abstract

Cell microencapsulation holds significant promise as a strategy for cellular therapies; however, inadequate survival and functionality of the enclosed cells limit its application in hemophilia treatment. Here, we evaluated the use of alginate-based microcapsules to enhance the viability and transgene secretion of human cord blood–derived mesenchymal stem cells in three-dimensional cultures. Given the positive effects of extracellular matrix molecules on mesenchymal stem cell growth, we tested whether fibrinogen-supplemented alginate microcapsules can improve the efficiency of encapsulated factor IX–engineered mesenchymal stem cells as a treatment of hemophilia B. We found that fibrinogen-supplemented alginate microcapsules (a) significantly enhanced the viability and proliferation of factor IX–engineered mesenchymal stem cells and (b) increased factor IX secretion by mesenchymal stem cells compared to mesenchymal stem cells in nonsupplemented microcapsules. Moreover, we observed the osteogenic, but not chondrogenic or adipogenic, differentiation capability of factor IX–engineered cord blood mesenchymal stem cells and their efficient factor IX secretion while encapsulated in fibrinogen-supplemented alginate microcapsules. Thus, the use of engineered mesenchymal stem cells encapsulated in fibrinogen-modified microcapsules may have potential application in the treatment of hemophilia or other protein deficiency diseases.

Keywords

mesenchymal stem cells, hemophilia, alginate, fibrinogen, cell encapsulation

Introduction

Hemophilia B is an X-linked bleeding disorder caused by human factor IX (hFIX) deficiency that occurs in 1 in 30,000 males.^{1,2} In the case of severe hemophilia, current treatment involves the lifelong and costly infusion of recombinant or plasma-derived factor IX (FIX) protein.³ Gene therapy offers an attractive alternative to current treatments because FIX expression is loosely regulated, and tissue-specific expression of a transgene is not required and the supply of as little as 1% of the physiological concentration of FIX has clinical benefits.⁴ Current gene therapy methods involve use of viral vectors for FIX delivery, which are associated with various safety issues; hence, development of an alternative method for FIX delivery is desirable.^{5–7} Here, we evaluated genetically engineered allogeneic mesenchymal stem cells (MSC) for their potential use in hemophilia B treatment.

Implantation of cells enclosed in biocompatible, semi-permeable microcapsules leads to continuous delivery of therapeutic proteins and protects cells from the host

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immune response.^{8–14} The host genome is not modified, and most of the microcapsules can be removed should it become necessary to reverse the treatment. As an additional safety measure, engineered cells can be thoroughly characterized for unwanted genetic rearrangements before encapsulation. To overcome the challenge of engineering cells from each individual, a universal cell line can be established and encapsulated in biocompatible microcapsules to be implanted into patients.

We previously described the use of alginate microcapsules enclosing recombinant fibroblasts, or C2C12 and G8 myoblasts, to deliver hFIX in mice.^{11–16} We also demonstrated that transplanted encapsulated cells play a key role in the immune response generated by the host against the transgene.¹³ It has been indicated that fetal myoblasts do not elicit the strong immune response to FIX seen in transformed myoblasts and therefore are more suitable for cell therapies.¹³ Transplantation of MSCs may also be a feasible strategy for the treatment of hemophilia B. MSCs are easy to culture, can be genetically manipulated to secrete bioactive molecules, are susceptible to molecules that modify their natural behavior, and have an immunosuppressive effect on immune cells involved in alloantigen recognition and elimination.^{17,18}

Biomaterial surfaces mimic the local microenvironment of cells by regulating cell attachment, viability, proliferation, migration, differentiation, and secretion of proteins. Alginate, the most commonly used biomaterial for cell encapsulation, does not provide sufficient cues for cell–matrix interactions.^{19–22} During cell encapsulation, anchorage-dependent MSCs encounter a lack of attachment and support. Current research in biomaterials and cell–biomaterial interactions focuses on compensating for this deficiency by providing the encapsulated cells with a microenvironment that more closely mimics their natural environment. One way to improve performance of the existing biomaterials is surface modification using bioactive molecules, such as native full-length extracellular matrix (ECM) proteins or the short-sequence peptides derived from them.²³ Optimally modified biomaterials would mimic the natural physiological environment of the encapsulated cells (“biomimetic”) and be readily available off the shelf. Among potential biomaterials available, several studies have addressed the significance of fibrinogen (a cell adhesion protein) in mediating cell survival, and different results have been reported, depending on the cell type used.^{20,24–26} In this study, we focus on the design of biomimetic microcapsules for cord blood (CB)-derived MSCs to monitor their viability, FIX secretion, and differentiation *in vitro*.

The objectives of the present study are (a) to assess the viability and FIX secretion level of umbilical CB-derived MSCs in three-dimensional (3D) alginate microcapsules; (b) to investigate whether by incorporating fibrinogen as a cell adhesion protein, alginate microcapsules can be manipulated

to increase viability, proliferation, and FIX secretion by CB MSCs; and (c) to induce differentiation of the encapsulated CB MSCs in fibrinogen–alginate microcapsules into osteogenic, chondrogenic, and adipogenic lineages in order to examine its effect on cell viability and FIX secretion.

Materials and methods

Cell culture

Umbilical CB was obtained after delivery with the mothers' informed consent in accordance with the guidelines of the University of Alberta Health Research Ethics Board. Light density mononuclear cells (MNC) were separated by Percoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and cultured in Iscove's modified Dulbecco's media (IMDM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum and streptomycin (100 µg/mL) at 37°C in 5% CO₂. After 24 h, nonadherent cells were removed and the complete medium was replaced, as described previously.²⁷ MSCs were used in experiments before reaching passage 6. Flow cytometric analysis showed positive expression for CD90 and CD105 but not for the hematopoietic markers CD34 and CD45 (data not shown).

Engineering of MSCs

The pLVX-Puro vector DNA (Clontech, Mountain View, CA, USA) was engineered with traditional restriction enzyme techniques to generate a FIX-expressing lentiviral DNA construct with cytomegalovirus (CMV) promoter. Viral particles were generated with the Lenti-X Expression System (Clontech) according to the manufacturer's protocol. Briefly, Lenti-X 293T cells were transfected with the fourth generation VSV-G packaging DNA and pLVX-FIXI expression plasmid using Xfect transfection reagent to generate viable virus particles. Two rounds of freshly produced viral supernatant were used to transduce CB MSCs (passage 4) for 24 h at a Multiplicity of Infection (MOI) of approximately 20. Transduced cells were selected with puromycin (3 µg/mL) (Clontech) after 15 days of incubation.

Cell encapsulation

Medium Viscosity High Guronate (MVC) ultrapure alginate was purchased from FMC BioPolymer (Philadelphia, PA, USA). FIX-engineered CB MSCs were suspended in a fibrinogen-supplemented and nonsupplemented (control) alginate solution (1.56% alginate) at a concentration of 3×10^6 cells/mL. Microencapsulation was performed with an electrostatic encapsulator (Nisco Engineering Inc., Zurich, Switzerland), as previously described.²⁸ Briefly, cell suspension was pumped through the electrostatic encapsulator (voltage: 7 kV) at the flow rate of 0.9 mL/min into a vial containing 1.1% CaCl₂

yielding microcapsules of 400 μm in diameter. Cell-loaded beads were then washed with saline solution and cross-linked with poly-L-lysine and with an outer layer of nonsupplemented alginate. Before encapsulation, human plasma fibrinogen (Sigma–Aldrich, Oakville, ON, Canada) was added to the alginate core as necessary at a concentration of 750 $\mu\text{g}/\text{mL}$ of alginate solution, as required.

For the monolayer studies, nonsupplemented (1.56% alginate) or fibrinogen-supplemented (750 mg fibrinogen/mL of 1.56% alginate) alginate mixture was placed over the sterilized coverslips. CaCl_2 was added drop-wise on the coverslips in order to gel the alginate. Coated coverslips were kept under the fume hood overnight and then seeded with CB–MSC and incubated overnight under normal tissue culture conditions.

Differentiation of human MSCs

For induction of osteogenic, chondrogenic, or adipogenic differentiation, encapsulated and nonencapsulated MSCs were cultured in StemPro Osteogenic, StemPro Chondrogenic, or StemPro Adipogenic differentiation media (GIBCO Invitrogen, Burlington, ON, Canada), respectively, and with appropriate supplements.

Nonencapsulated cells were grown in differentiation media in monolayer cultures and stained for differentiation markers. At week 3 of postosteogenic induction and at week 2 of postchondrogenic and postadipogenic inductions, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. The cells were stained with Alizarin Red, Alcian Blue, and Oil Red O dyes (Sigma–Aldrich) for detection of calcium deposits, proteoglycans, and fat vacuoles as an indication of osteogenic, chondrogenic, and adipogenic differentiations, respectively. The cells were visualized under an inverted light microscope (Leica DM IL; Leica Microsystems, Richmond Hill, ON, Canada).

Assessment of viability and proliferation of encapsulated cells

Viability of encapsulated MSCs was assessed using the trypan blue exclusion assay. Proliferation of encapsulated MSCs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma–Aldrich, Oakville, ON, Canada) according to the manufacturer's instructions adapted to encapsulated cells. Briefly, 100 μL of microcapsules were incubated with 10 μL MTT reagent in 96-well plate for 3 h at 37°C. After 3 h, when the intracellular punctate (purple precipitate) was clearly visible under the microscope, the resultant formazan was dissolved in 100 μL dimethyl sulfoxide (DMSO; BDH Inc., Toronto, ON, Canada). The plate was incubated in the dark for 3 h at room temperature and absorbance measured at 562 nm (EL 808 Ultra Microplate Reader; Bio-Tek Instruments Inc., Winooski, VT, USA).

F-actin cytoskeleton staining of encapsulated cells

Encapsulated cells (100 μL of capsules) were fixed in 4% paraformaldehyde. As indicated in the manufacturer's protocol, cells were then washed in prewarmed PBS and permeabilized by incubation in 0.1% Triton X-100 in PBS for 10 min, followed by two washes in PBS. Cells were stained with Alexa Fluor 633 phalloidin (15 μL methanolic stock solution in 200 μL PBS, for 30 min in the dark) (Molecular Probes Invitrogen, Burlington, ON, Canada) containing 1% bovine serum albumin to reduce nonspecific background. A sample was analyzed by inverted confocal microscopy (Zeiss 510; Carl Zeiss Inc., Toronto, ON, Canada).

Transmission electron microscopy and scanning electron microscopy

All transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies were done at the electron microscopy facility at McMaster Children's Hospital (Hamilton, ON, Canada). Encapsulated cells were fixed with 2% glutaraldehyde (2% v/v) in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were rinsed twice in buffer solution, then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, and dehydrated through a graded ethanol (EtOH) series (50%–100%). For TEM analysis, the final dehydration of the TEM samples was done in 100% propylene oxide (PO). Infiltration with Spurr's resin was done through a graded series of PO:Spurr 2:1, 1:1, 1:2, and 0:1 with rotation of the samples in between solution changes. The samples were transferred to embedding moulds that were then filled with fresh 100% Spurr's resin and polymerized overnight in a 60°C oven. Sections were cut on a Leica UCT ultramicrotome (Leica Microsystems, Richmond Hill, ON, Canada) and picked up onto copper grids. The sections were poststained with uranyl acetate and lead citrate and viewed in a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80 kV. For SEM analysis, after dehydration in 100% EtOH, some capsules were frozen, fractured, and placed back into 100% EtOH to thaw. Samples were then dried at critical point, mounted onto SEM stubs, sputter-coated with gold, and viewed in a Tescan VEGA II LSU scanning electron microscope (Tescan USA, Cranberry Township, PA, USA) at 20 kV.

FIX enzyme-linked immunosorbent assay

Human FIX antigen in culture media was quantified by an enzyme-linked immunosorbent assay (ELISA) (Affinity Biologicals Inc., Ancaster, ON, Canada), as previously described.¹³

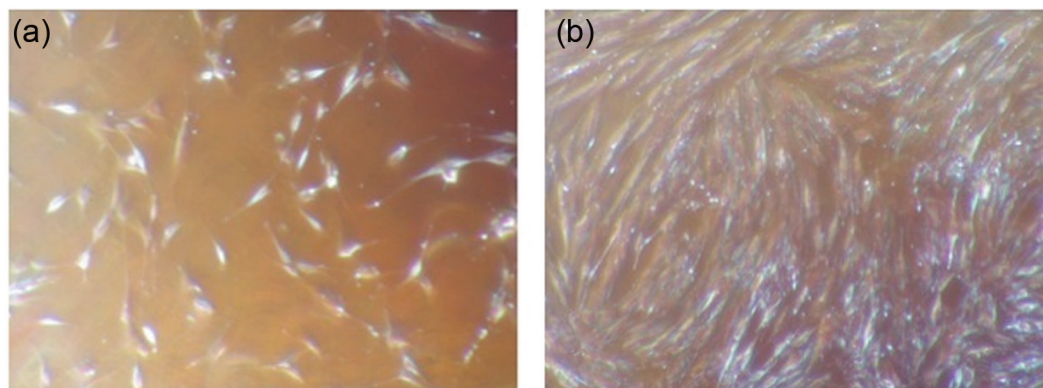


Figure 1. Effect of fibrinogen-supplemented alginate on proliferation of FIX-engineered CB MSCs. Light microscopy images ($\times 10$) of MSC grown in monolayer at 24 h post culture on (a) nonsupplemented alginate-coated coverslip and (b) fibrinogen-supplemented alginate-coated coverslip.

FIX: factor IX; CB: cord blood; MSC: mesenchymal stem cell.

Animal studies

All animal procedures were conducted in accordance with the Animal Ethics Guidelines of McMaster University. C57BL/6 mice were purchased from Charles River (Montreal, QC, Canada) and maintained at the Central Animal Facility, McMaster University (Hamilton, ON, Canada). The mice were anesthetized with isoflurane (Bimeda-MTC Animal Health Inc., Richmond Hill, ON, Canada) in a small animal anesthetic machine (Med-Vet Anaesthetic System Inc., Toronto, ON, Canada). Each mouse received 2 mL of microcapsules intraperitoneally using a G18 catheter.

Statistical data analysis

Analysis of variance (ANOVA) was carried out to determine whether significant differences existed in the groups of data. Student's *t* test was conducted as a post hoc test to compare the pairs of data. Differences were considered significant when $p < 0.05$. Data are expressed as means \pm standard deviation (SD).

Results

Determining cell–matrix interactions in monolayer

In order to assess the effect of fibrinogen on the morphology and proliferation of FIX-engineered CB MSCs, 3×10^6 cells were cultured in monolayer overnight on coverslips coated with nonsupplemented alginate (control) or fibrinogen-supplemented alginate. The presence of fibrinogen resulted in enhanced proliferation of MSCs (Figure 1).

Assessment of MSC viability and proliferation in microcapsules

To determine the proliferation and FIX secretion of MSCs in fibrinogen-supplemented and nonsupplemented (control)

alginate microcapsules, encapsulated cells were cultured *in vitro* for 28 days. The MTT assay confirmed that fibrinogen significantly increased the viability of encapsulated cells over control capsules (Figure 2(a)). The trypan blue assay showed no significant difference on day 1 (82% in the control group and 79% in the fibrinogen-supplemented group). Viability dropped to 55% by day 28 in control microcapsules but remained high (72%) in fibrinogen microcapsules. Additionally, an MTT assay confirmed that cell proliferation was significantly higher in fibrinogen–alginate microcapsules than in alginate microcapsules throughout the 28 days and was 35% higher on day 28 (Figure 2(b)).

FIX secretion from encapsulated cells

Aside from stable proliferation, a sustained FIX secretion is of paramount importance. Consistent with the significantly higher viability and proliferation of MSCs, incorporation of fibrinogen in the alginate matrix resulted in higher FIX secretion from the encapsulated MSCs throughout the experiment, as measured by ELISA. The average FIX secretion from fibrinogen-supplemented device was above 4000 ng/mL (capsules/24 h) during the first 2 weeks of *in vitro* growth (Figure 3). In agreement with the trend in cell proliferation (Figure 2), FIX secretion dropped to above 2000 ng/mL (capsules/24 h) in cells in fibrinogen–alginate microcapsules and to ~ 1000 ng/mL (capsules/24 h) in control microcapsules on day 26 (Figure 3).

Cell–matrix analysis

For an in-depth, 3D look at the cell–matrix interaction, we examined encapsulated MSCs using confocal microscopy, TEM, and SEM (Figure 4). As detected by TEM, fibrinogen cells clearly demonstrated the presence of filopodia-like membrane extensions into the surrounding matrix (Figure 4(a) and (b)), which were lacking in control microcapsules (Figure

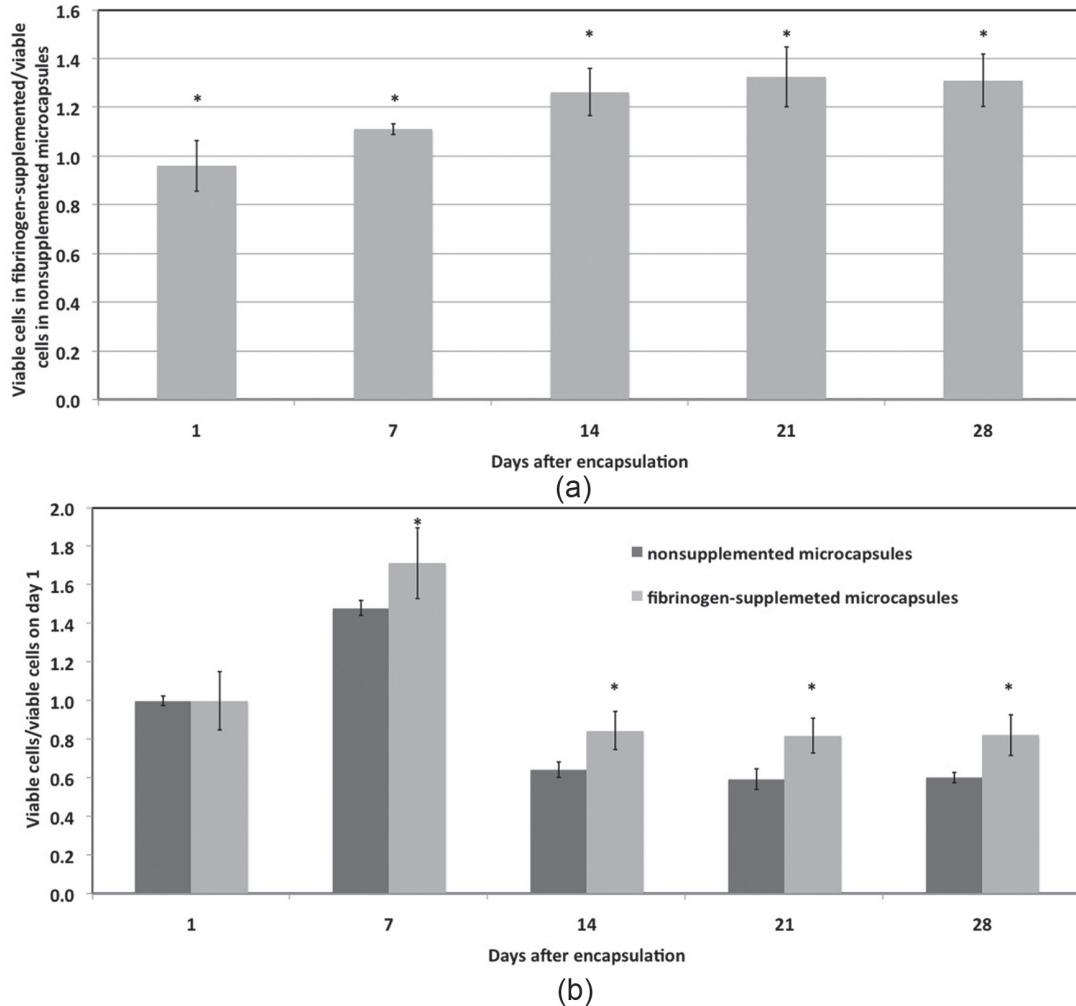


Figure 2. Viability and proliferation of CB MSCs: (a) the effect of fibrinogen–alginate microcapsules on viability of encapsulated cells. The ratio of viable cells in fibrinogen-supplemented microcapsules to the viable cells in nonsupplemented microcapsules was calculated using the MTT assay. (b) Comparison of MSC proliferation in nonsupplemented and fibrinogen-supplemented microcapsules. The ratio of viable cells per viable cells at day 1 was calculated using the MTT assay. Data are mean \pm SD, $n = 3$, Student's t test. *Significant difference from 1.000, $p < 0.09$.

CB: cord blood; MSC: mesenchymal stem cell; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD: standard deviation.

4(c) and (d)). However, SEM and confocal analysis did not reveal significant differences between both groups in terms of morphology or F-actin staining, SEM: Figure 4(e) and (f) versus (g) and (h) and confocal: Figure 4(i) and (j) versus (k) and (l). Patterns or alignments of cellular filaments were not detectable in either group of microcapsules by confocal microscopy (Figure 4(i) to (l)). Furthermore, SEM images did not show any obvious differences in cell morphology between either types of microcapsules (Figure 4(e) to (h)).

Effect of differentiation induction on encapsulated cell viability, proliferation, and FIX secretion

To investigate the effect of viral transduction on the differentiation potential of MSCs, FIX-engineered MSCs were

grown in monolayer cultures and induced to differentiate into osteogenic, chondrogenic, and adipogenic lineages. FIX-engineered MSCs successfully differentiated into osteocytes within 3 weeks (Figure 5(a)) and chondrocytes within 2 weeks (Figure 5(b)), but not adipocytes (Figure 5(c)). The lack of adipogenic differentiation was consistent with the previous reports of CB MSCs.²⁹ Comparable differentiation properties were observed in nontransduced MSCs. Our results show that transduction of MSCs with lentivirus did not affect their differentiation potential.

Furthermore, the effect of the differentiation induction on cell viability and proliferation was assessed. MSCs in fibrinogen–alginate microcapsules were cultured in one of adipogenic, chondrogenic, or osteogenic differentiation media or in control basal medium. The total numbers of

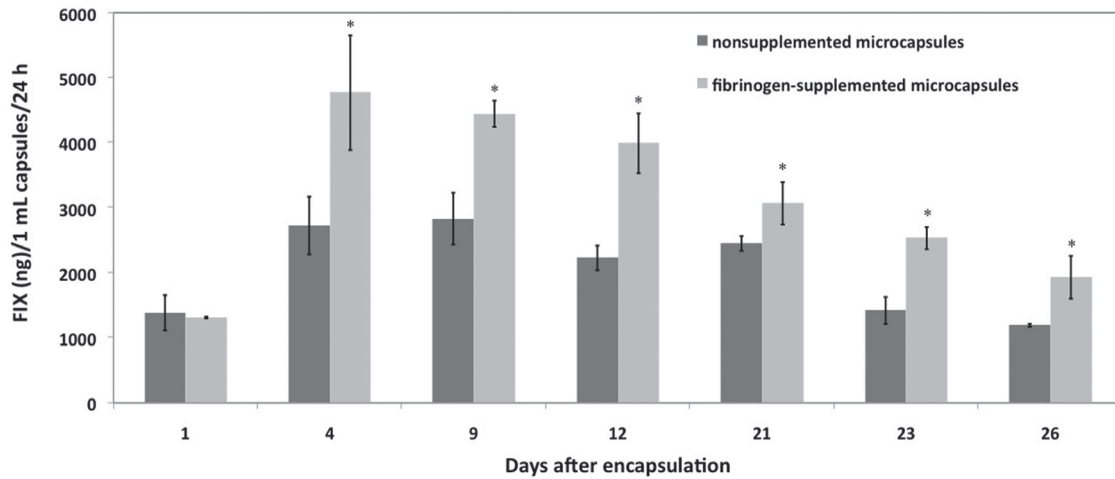


Figure 3. FIX secretion by CB MSCs. Comparison of FIX secretion from MSCs in nonsupplemented and fibrinogen-supplemented microcapsules. FIX secretion was measured using ELISA assay and reported as the amount of FIX (ng) secreted from 1 mL of microcapsules in 24 h. Data are means \pm SD, $n = 4$, $p < 0.05$, Student's t test. *Significant difference: nonsupplemented microcapsules versus fibrinogen-supplemented microcapsules on each day.

CB: cord blood; MSC: mesenchymal stem cell; FIX: factor IX; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation.

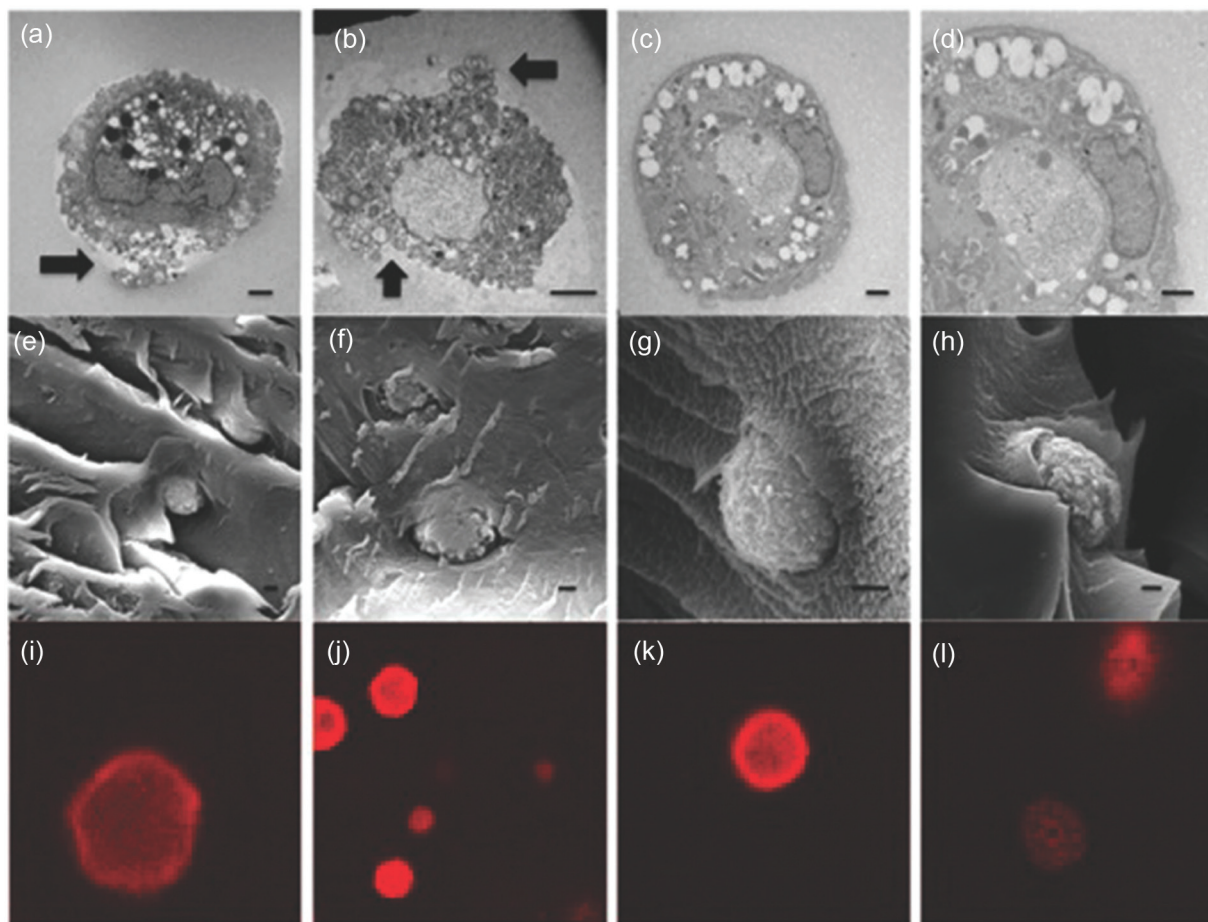


Figure 4. (a to d) Transmission electron microscopy images of CB MSCs encapsulated in (a, b) fibrinogen-supplemented alginate microcapsules or (c, d) nonsupplemented alginate microcapsules. (e to h) Scanning electron microscopy images of CB MSCs encapsulated in (e, f) fibrinogen–alginate microcapsules or (g, h) nonsupplemented alginate microcapsules. (i to l) F-actin staining of CB MSCs encapsulated in (i, j) fibrinogen–alginate microcapsules or (k, l) nonsupplemented alginate microcapsules. Cells were fixed 7 days after encapsulation and processed for electron and confocal microscopy. Bars indicate 2 μ m.

CB: cord blood; MSC: mesenchymal stem cell.

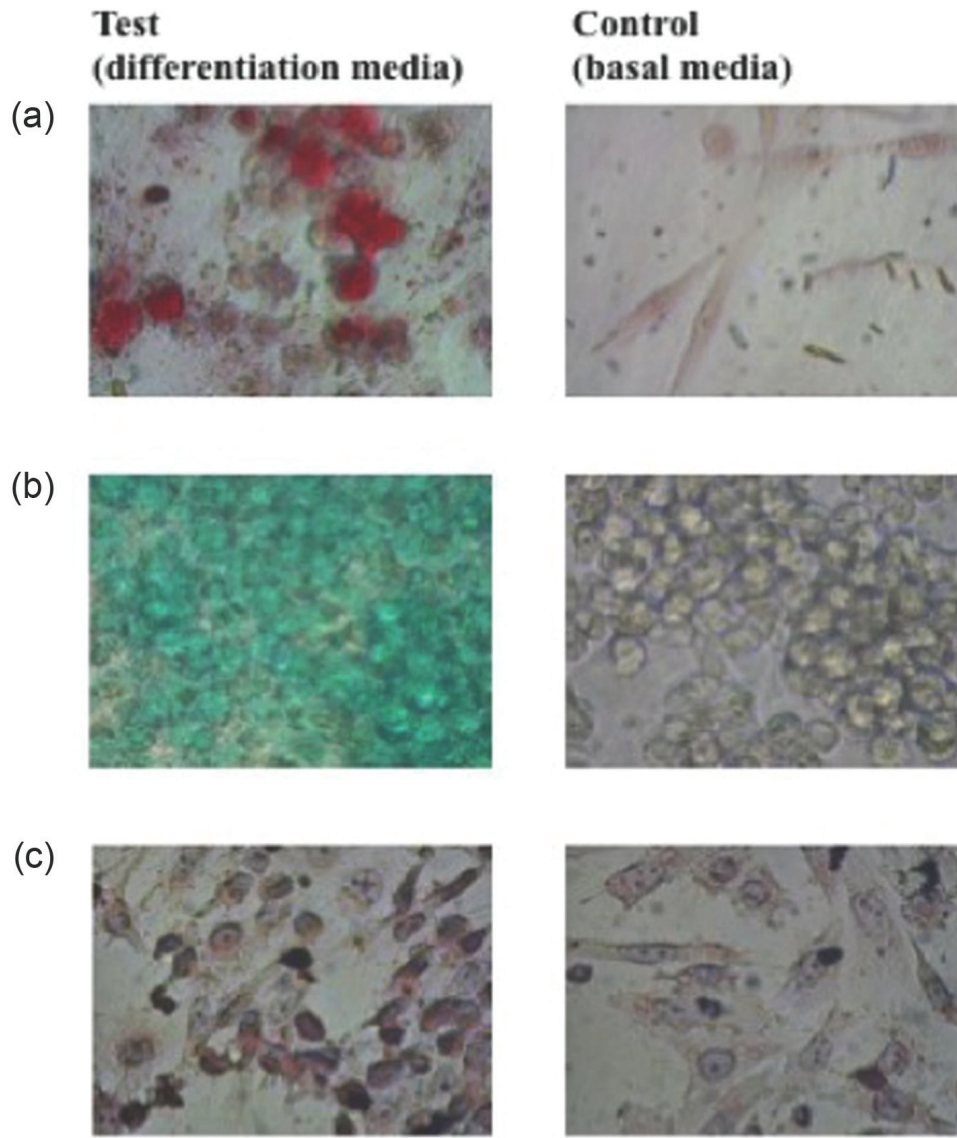


Figure 5. Differentiation potential of FIX-engineered CB MSCs: (a) osteogenic differentiation, (b) chondrogenic differentiation, and (c) adipogenic differentiation.

FIX: factor IX; CB: cord blood; MSC: mesenchymal stem cell.

viable cells in differentiation media were normalized with respect to the total number of viable cells in basal medium (Figure 6(a)). The viability of cells grown in osteogenic medium was not significantly different from the viability of cells grown in basal medium and was significantly higher than cells grown in chondrogenic and adipogenic media. A similar trend was also observed in cellular proliferation in different media during 28 days in vitro (Figure 6(b)). The low viability and proliferation of encapsulated cells grown in chondrogenic and adipogenic media suggest the inability of the proposed fibrinogen–alginate microcapsule system to support chondrogenic and adipogenic differentiations.

The effect of differentiation induction on FIX secretion was also investigated. During the first 2 weeks of culture, FIX secretion from encapsulated cells cultured in basal media was significantly higher than the secretion from cells grown in osteogenic, chondrogenic, or adipogenic media (Figure 7). By the third week, consistent with the differentiation time of MSCs into osteoblast (21 days, osteogenic differentiation protocol, GIBCO Invitrogen), FIX secretion from the cells grown in basal and osteogenic media was significantly higher than the secretion from cells grown in chondrogenic or adipogenic media. The low FIX secretion from cells grown in chondrogenic and adipogenic media was consistent with the low

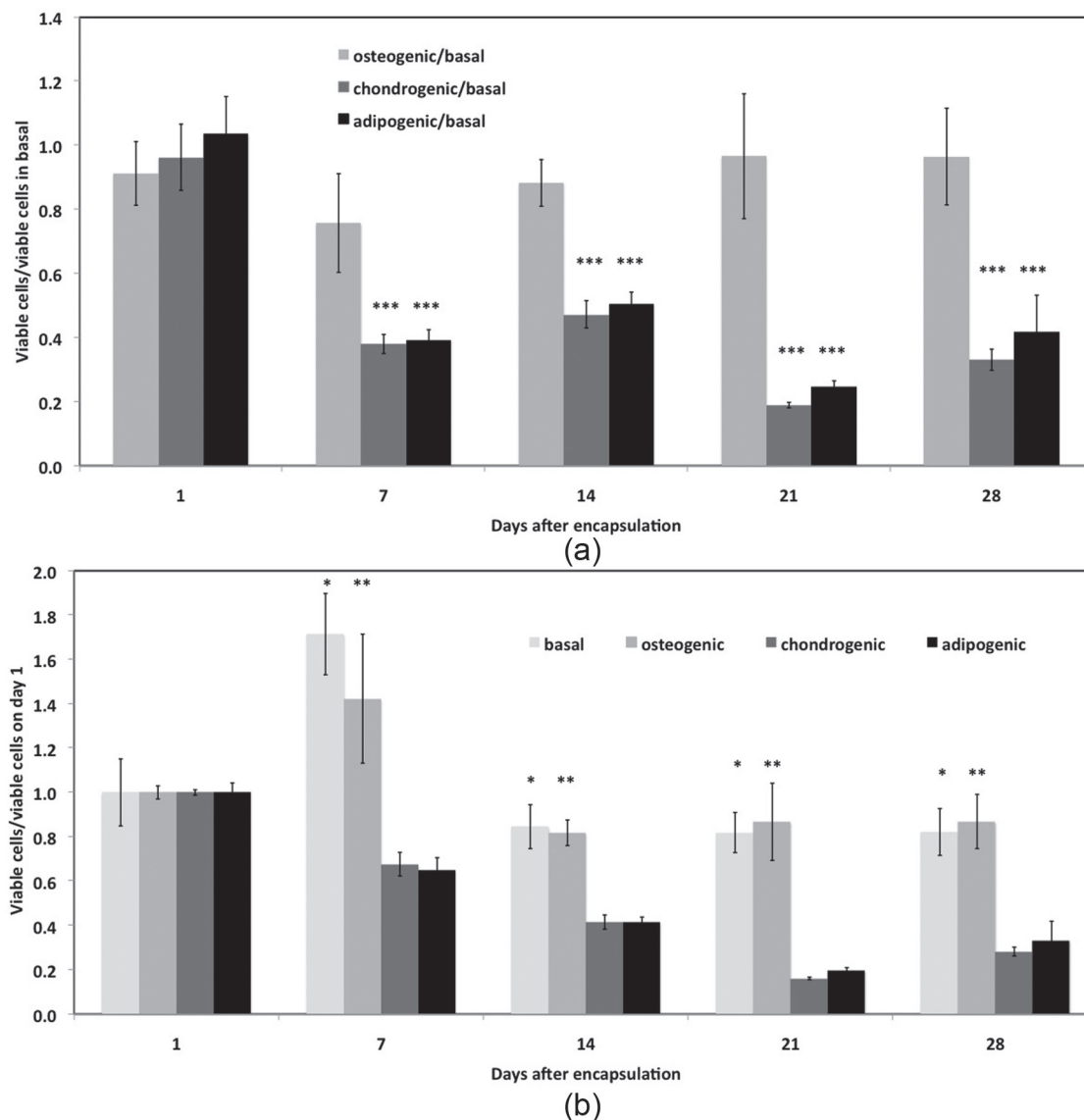


Figure 6. Viability and proliferation of CB MSCs. (a) MSC viability in fibrinogen-supplemented microcapsules cultured in differentiation media normalized to the viability of cells grown in basal media. (b) Comparison of MSC proliferation in fibrinogen-supplemented microcapsules grown in basal and differentiation media. Data are means \pm SD, $n = 3$. The ratio of viable cells per viable cells at day 1 was calculated using the MTT assay. $p < 0.05$, Student's t test. *Significant difference test-basal versus test-chondrogenic and test-adipogenic. **Significant difference test-osteogenic versus test-chondrogenic and test-adipogenic. ***Significant difference from 1.000.

CB: cord blood; MSC: mesenchymal stem cell; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD: standard deviation.

viability and proliferation observed for the cells grown in these conditions.

Biocompatibility of fibrinogen-supplemented alginate microcapsules in vivo

To further assess the biocompatibility of the fibrinogen-supplemented microcapsules, fibrinogen-supplemented and nonsupplemented alginate microcapsules were injected without cells intraperitoneally in C57BL/6 mice. All mice were sacrificed on day 7 post treatment, and implanted

capsules were retrieved and analyzed by inverted light microscopy (Figure 8). The biocompatibility of both types of microcapsules was similar. There was no obvious overgrowth around the fibrinogen-supplemented microcapsules, suggesting good biocompatibility.

To monitor fibrinogen leakage, 2 mL microcapsules were suspended in 10 mL PBS, and the regular supernatant samples were analyzed for evidence of fibrinogen using ultraviolet (UV) spectroscopy at 280 nm. As observed by the UV spectroscopy, fibrinogen leakage was undetectable throughout the experiment (with a minimum

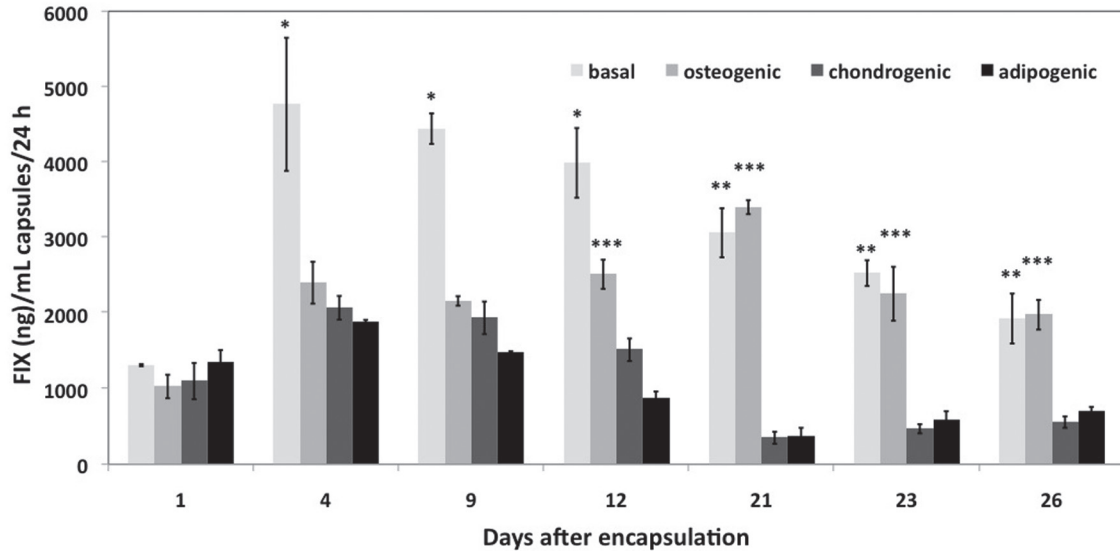


Figure 7. FIX secretion of CB MSCs. Comparison of FIX secretion from the MSCs in fibrinogen-supplemented microcapsules grown in differentiation media (data are means \pm SD, $n = 4$). FIX secretion was measured using ELISA. $p < 0.05$, Student's t test. *Significant difference fibrinogen–alginate (basal) versus all other conditions. **Significant difference fibrinogen–alginate (basal) versus fibrinogen–alginate (chondrogenic) and fibrinogen–alginate (adipogenic). ***Significant difference fibrinogen–alginate (osteogenic) versus fibrinogen–alginate (chondrogenic) and fibrinogen–alginate (adipogenic). CB: cord blood; MSC: mesenchymal stem cell; FIX: factor IX; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation.

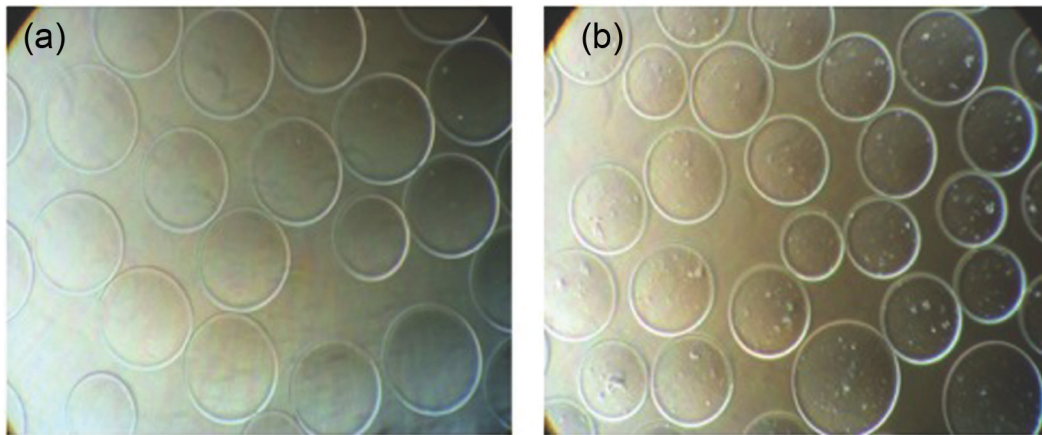


Figure 8. Empty capsules retrieved from mice after 7 days in vivo: (a) nonsupplemented alginate–PLL–alginate microcapsules and (b) fibrinogen-supplemented alginate–PLL–alginate microcapsules (no significant overgrowth around microcapsules). PLL: poly-L-lysine.

level of detection of $0.5 \mu\text{g/mL}$; data not shown). This is consistent with the good biocompatibility observed in mice (Figure 8).

Discussion

The purpose of this study was to investigate the viability, proliferation, and FIX secretion of engineered CB MSCs encapsulated in alginate–fibrinogen microcapsules. Previous

studies showed that biomimetic arginine–glycine–aspartic acid (RGD)–alginate microcapsules improved the long-term viability and functionality of encapsulated C2C12 myoblasts but to the best of our knowledge, this is the first time fibrinogen–alginate capsules have been used for the encapsulation of MSC.³⁰ Our data indicate that incorporation of fibrinogen enhances cell viability and proliferation, as well as FIX secretion of MSCs.

The improved proliferation of MSC in fibrinogen–alginate versus nonsupplemented alginate microcapsules is in

agreement with the results obtained from our monolayer cultures (Figure 1). Although further studies are required to analyze the mechanism of cell–matrix interactions, fibrinogen may provide matrix cues that the MSCs interact with. MSCs are able to attach to fibrinogen through several integrins,^{31–33} and it is conceivable that the enhancement of MSC proliferation and viability observed in this study is modulated by MSCs interacting directly with fibrinogen. Integrins activate survival pathways via the phosphoinositide-3-kinase (PI-3-kinase) and mitogen-activated protein kinase (MAPK) pathways and act as essential cofactors for MSC stimulation by growth factors.²⁰ Integrins may activate the MAPK pathway by either of two cascades, one of which involves focal adhesion kinase (FAK) and the other the adaptor protein Shc.²⁶ Karoubi et al.²⁴ reported that increased viability of encapsulated MSCs in fibrinogen and fibronectin-supplemented agarose is likely via the Shc signaling pathway, which is an essential intermediate of the MAPK cascade.²⁴ It has also been demonstrated that $\alpha_v\beta_3$ and $\alpha_5\beta_1$, the binding partner integrins of fibrinogen, are among the few integrins that recruit and activate Shc and, thus, play a critical role in the survival of adherent MSC.^{19,20}

Improved viability of encapsulated MSCs in fibrinogen–alginate microcapsules may potentially result from the reinduction of the cell–matrix interaction via the activation of the extracellular signal-regulated MAPK cascade. Additionally, it was previously reported that once the MSC reaches local confluence, the rate of proliferation decreases from log phase growth.³⁴ The acellular regions in nonsupplemented alginate-covered coverslips were likely due to discontinuous and colony-like proliferation patterns. Conversely, the near homogeneous coverage observed in fibrinogen–alginate-covered coverslips suggests a better distribution of cells during proliferation. MSCs were also more homogenous during growth in fibrinogen–alginate microcapsules compared to nonsupplemented microcapsules (data not shown). The increased dispersal of MSCs during proliferation may explain, in part, their higher viability and proliferation in fibrinogen-supplemented alginate. Contact inhibition, which slows cell growth, may be reduced in supplemented microcapsules.³³ The filopodia-like extensions observed by TEM in the cells encapsulated in fibrinogen–alginate may suggest enhanced attachment. However, such evidence was undetectable by SEM or confocal microscopy at the fibrinogen concentration used.

FIX is a clotting factor in the coagulation cascade. Hence, bioavailability of exogenous FIX and fibrinogen in the microcapsules may promote a thrombogenic state, thus posing a safety concern. However, and despite the accepted risk for thrombosis in patients with supraphysiological FIX levels, gene therapy strategies achieving 30 times the normal FIX level did not cause thrombosis in mice.³⁵ Importantly, the concentration of fibrinogen used in the microcapsules (750 $\mu\text{g}/\text{mL}$) is modest when compared to physiological levels of fibrinogen (10 mg/mL). Furthermore, fibrinogen is unable to

lead to fibrin formation without a higher bioavailability of thrombin, and hemophilic mice implanted with myoblasts secreting similar amounts of FIX *in vitro* as MSC did not elicit a thrombotic state, suggesting no additional production of thrombin.¹³ Additionally, UV spectroscopy analysis revealed no detectable leakage of fibrinogen from microcapsules. Finally, we established the biocompatibility of the fibrinogen–alginate microcapsules in mice, with no obvious signs of cell overgrowth or immune response, thus supporting the notion that no fibrinogen leaked from the microcapsules.

We have previously reported the therapeutic secretion of FIX in hemophilic mice implanted with microcapsules containing G8 myoblasts secreting *in vitro* ~1500 ng FIX/ 10^6 cells/24 h.¹³ Of note, this level is comparable to the FIX secreted by CB MSCs in this study, thus suggesting the therapeutic potential of encapsulated MSCs. Future *in vivo* studies are warranted to determine the potential of encapsulated MSCs in the treatment of hemophilia.

Our differentiation experiments demonstrate that encapsulated cells cultured in osteogenic and basal media exhibit increased viability and proliferation, and consequently enhanced FIX secretion, compared with encapsulated cells grown in chondrogenic and adipogenic cultures. Monolayer studies showed that FIX transduction of CB MSCs did not affect the multipotency of cells grown in monolayer culture. Also, CB MSCs differentiated through mesenchymal lineages only when induced by differentiation media. Understanding the effect of differentiation induction on encapsulated cell viability and functionality would allow modulation of cell differentiation status before implantation for optimum functionality. We showed that high viability, proliferation, and FIX secretion of encapsulated cells grown in basal and osteogenic media were maintained for at least 28 days. However, cell viability, proliferation, and FIX secretion were lower in chondrogenic and adipogenic cultures, suggesting the inability of the microcapsule system to support chondrogenic and adipogenic differentiations. Consistently, it has been reported that cell aggregates are needed to induce chondrogenic differentiation in MSCs in 3D cultures.³⁶ Moreover, the low cell viability in adipogenic media was predicted from our monolayer differentiation studies (Figure 5) and was expected due to the known inefficiency of CB MSCs to differentiate into the adipogenic lineage.²⁹

Conclusions

Encapsulation of recombinant cells holds significant promise for treatment of diseases caused by protein deficiencies, such as hemophilia B. The availability and ease of manipulation of MSCs make them particularly attractive for future cellular therapies. However, the viability and functionality of the enclosed cells are still major challenges of cell encapsulation. Bioactive fibrinogen–alginate microcapsules

examined in this study enhanced cell viability and FIX secretion from the enclosed FIX-engineered MSCs. Moreover, fibrinogen–alginate microcapsules provided an appropriate environment for osteogenic differentiation. Fibrinogen–alginate microencapsulation of FIX-engineered MSCs has potential for cell-based gene therapy of hemophilia B and other inherited or acquired protein deficiencies. However, further studies should address the mechanisms behind improved MSC viability and functionality, as well as the characterization of the biological interactions of MSCs with the fibrinogen–alginate matrix.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

- Sadler J and Davie E. The metabolic basis of inherited disease. In: Nienhuis AW, Leder P and Majerus PW (eds) *Hemophilia A, hemophilia B, and Von Willebrand's disease*. New York: W. B. Saunders, 1987, pp. 575–598.
- Bolton-Maggs PHB and Pasi KJ. Haemophilias A and B. *Lancet* 2003; 361(9371): 1801–1809.
- Gater A, Thomson TA and Strandberg-Larsen M. Haemophilia B: impact on patients and economic burden of disease. *Thromb Haemost* 2011; 106(3): 398–404.
- Manno CS, Chew AJ, Hutchison S, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; 101(8): 2963–2972.
- Nathwani AC, Tuddenham EGD, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*, <http://www.ncbi.nlm.nih.gov/pubmed/22149959> (2011, accessed 21 December 2011).
- Marshall E. Gene therapy. Panel reviews risks of germ line changes. *Science* 2001; 294(5550): 2268–2269.
- Check E. Harmful potential of viral vectors fuels doubts over gene therapy. *Nature* 2003; 423(6940): 573–574.
- Orive G, Hernández RM, Gascón AR, et al. Cell encapsulation: promise and progress. *Nat Med* 2003; 9(1): 104–107.
- Orive G, Tam SK, Pedraz JL, et al. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. *Biomaterials* 2006; 27(20): 3691–3700.
- Thakur A, Sengupta R, Matsui H, et al. Characterization of viability and proliferation of alginate-poly-L-lysine-alginate encapsulated myoblasts using flow cytometry. *J Biomed Mater Res B Appl Biomater* 2010; 94(2): 296–304.
- Hortelano G, Al-Hendy A, Ofofu FA, et al. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood* 1996; 87(12): 5095–5103.
- Wen J, Xu N, Li A, et al. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *J Gene Med* 2007; 9(11): 1002–1010.
- Wen J, Vargas AG, Ofofu FA, et al. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *J Gene Med* 2006; 8(3): 362–369.
- Hortelano G, Wang L, Xu N, et al. Sustained and therapeutic delivery of factor IX in nude haemophilia B mice by encapsulated C2C12 myoblasts: concurrent tumorigenesis. *Haemophilia* 2001; 7(2): 207–214.
- Hortelano G, Xu N, Vandenberg A, et al. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. *Hum Gene Ther* 1999; 10(8): 1281–1288.
- Hortelano G and Chang PL. Gene therapy for hemophilia. *Artif Cells Blood Substit Immobil Biotechnol* 2000; 28(1): 1–24.
- Liu M and Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *J Cell Mol Med* 2008; 12(4): 1155–1168.
- Frisch SM and Ruoslahti E. Integrins and anoikis. *Curr Opin Cell Biol* 1997; 9(5): 701–706.
- Zvibel I, Smets F and Soriano H. Anoikis: roadblock to cell transplantation? *Cell Transplant* 2002; 11(7): 621–630.
- Stupack DG and Chersesh DA. Get a ligand, get a life: integrins, signaling and cell survival. *J Cell Sci* 2002; 115(Pt 19): 3729–3738.
- Valentijn AJ, Zouq N and Gilmore AP. Anoikis. *Biochem Soc Trans* 2004; 32(Pt 3): 421–425.
- Grossmann J. Molecular mechanisms of “detachment-induced apoptosis—anoikis.” *Apoptosis* 2002; 7(3): 247–260.
- Shin H, Jo S and Mikos AG. Biomimetic materials for tissue engineering. *Biomaterials* 2003; 24(24): 4353–4364.
- Karoubi G, Ormiston ML, Stewart DJ, et al. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials* 2009; 30(29): 5445–5455.
- Stupack DG, Puente XS, Boutsaboualoy S, et al. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 2001; 155(3): 459–470.
- Whitlock BB, Gardai S, Fadok V, et al. Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *J Cell Biol* 2000; 151(6): 1305–1320.
- Son B-R, Marquez-Curtis LA, Kucia M, et al. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells* 2006; 24(5): 1254–1264.

28. Chang PL, Hortelano G, Awrey DE, et al. Growth of recombinant fibroblasts in alginate microcapsules. *Biotechnol Bioeng* 1994; 43(10): 925–933.
29. Rebelatto CK, Aguiar AM, Moretão MP, et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Exp Biol Med (Maywood)* 2008; 233(7): 901–913.
30. Orive G, De Castro M, Kong H-J, et al. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *J Control Release* 2009; 135(3): 203–210.
31. Gailit J, Clarke C, Newman D, et al. Human fibroblasts bind directly to fibrinogen at RGD sites through integrin alpha(v) beta3. *Exp Cell Res* 1997; 232(1): 118–126.
32. Schmal H, Niemeyer P, Roesslein M, et al. Comparison of cellular functionality of human mesenchymal stromal cells and PBMC. *Cytotherapy* 2007; 9(1): 69–79.
33. Kisiday JD, Hale BW, Almodovar JL, et al. Expansion of mesenchymal stem cells on fibrinogen-rich protein surfaces derived from blood plasma. *J Tissue Eng Regen Med*, <http://www.ncbi.nlm.nih.gov/pubmed/21774083> (2010, accessed 8 June 2011).
34. Neuhuber B, Swanger SA, Howard L, et al. Effects of plating density and culture time on bone marrow stromal cell characteristics. *Exp Hematol* 2008; 36(9): 1176–1185.
35. Ehrhardt A and Kay MA. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood* 2002; 99(11): 3923–3930.
36. Goren A, Dahan N, Goren E, et al. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. *FASEB J* 2010; 24(1): 22–31.