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Research Article

Proliferation and Differentiation of Mesenchymal Stem Cells Encapsulated in Miniaturized 3D Core of Alginate-Chitosan-Alginate (ACA) Microcapsules

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Abstract

Murine C3H10T1/2 cells were used as a model of mesenchymal stem cells (MSCs) in this study to investigate their capability of proliferation and differentiation in the miniaturized core of small (~100 μm) alginate-chitosan-alginate (ACA) microcapsules. The results show that the cells can survive and proliferate well in the microcapsules, particularly those with a liquid core. Moreover, successful adipogenic, osteogenic, and chondrogenic differentiation of the encapsulated C3H10T1/2 cells were confirmed. Collectively, the small ACA microcapsules are a promising carrier for MSC-based medicine.

INTRODUCTION

Transplantation of encapsulated cells is a promising approach for the treatment of a wide variety of diseases because it prevents the encapsulated cells from undesired immune rejection and enables controlled and continuous delivery of therapeutic agents produced by the cells [1-8]. Moreover, culturing in microcapsules also provides a miniaturized three-dimensional (3D) micro environment for the proliferation and differentiation of stem cells [5]. Mesenchymal stem cells (MSCs) are multi-potent cells that could be guided/directed to differentiate into osteoblasts, myocytes, chondrocytes, and adipocytes as well as insulin-producing cells [9]. Recently, encapsulation of MSCs (or genetically modified MSCs) from different resources in alginate-based microspheres/capsules have been studied for bone tissue engineering, regenerative therapies for cardiovascular diseases, and treatment of hemophilia or other diseases due to protein deficiency [6,10-20]. Efforts have also been made for eventually initiating clinical applications of this technique, such as ongoing safety trials on human of CellBead® technology [21] forcell-based regenerative medicine. Interestingly, microencapsulation of cells has also shown to be important for improving the outcomes of cry preserving MSCs [3,4].

The small (~100 μm) alginate-chitosan-alginate (ACA) microcapsule system was developed and shown to be a promising carrier for stem cells in our previous study [5]. Small microcapsules offer many advantages over larger ones for transplantation in terms of biotransport of nutrients and oxygen and feasibility for transplantation [2,5]. The C3H10T1/2 cells were derived from mouse embryo cells and could be differentiated into various lineages including osteoblasts and chondrocytes [6,22]. Therefore, they could be a good alternative source of primary mesenchymal stem cells (MSCs). In this study, the proliferation and differentiation of C3H10T1/2cells cultured in the miniaturized 3D core of small ACA microcapsules were studied.

MATERIALS AND METHODS

Materials

Purified sodium alginate (type M) was purchased from Medipol (Lausanne, Switzerland). Chitosan of pharmaceutical grade (80 kDa and~95% deacetylation) was obtained from Weikang Biological Products Co. Ltd (Shanghai, China). The live/dead viability or cytotoxicity kit for mammalian cells, trypsin/EDTA, and regular DMEM (high glucose) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum, penicillin, and streptomycin were purchased from Hyclone (Logan, Utah). All

other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specifically mentioned otherwise.

Cell Culture

C3H10T1/2 mesenchymal stem cells (MSCs)from ATCC (Manassas, VA) were cultured in regular DMEM with 10% fetal bovine serum, 100 U ml^{-1}penicillin,and 100 mg l^{-1}streptomycin. On the day of experiments, the cells in culture were collected by detaching them from culture dishes or flasks with trypsin/EDTA digestion, centrifuged for 3 minutes at 960 rpm, and re-suspended in a 0.9% (w/v) sodium chloride solution (physiological saline) for further experimental use.

Cell Encapsulation

Encapsulation of C3H10T1/2 MSCs into small (~ 100 μm) alginate-chitosan-alginate (ACA) microcapsules was conducted using a procedure detailed elsewhere [5]. Briefly, cells were mixed with a 2.25% (w/v) sodium alginate solution (prepared using 0.25 M mannitol solution buffered by 10 mM HEPES) to obtain a cell/alginate mixture, where the final cell density is 4.5 million cells/ml and the final alginate concentrationis 2% (w/v). The mixture was sprayed into 150 mM $CaCl_2$ solution to obtain plain alginate microbeads. The plain alginate microbeads were then collected by slight centrifugation (500 rpm for 3 min) and washed using 0.5 M mannitol (in physiologic saline) for 6 minutes and suspended in chitosan (0.4%w/v in physiologic saline) solution (pH 6.5–6.6) for 2 minutes to obtain the alginate-chitosan (AC) microcapsules. After collecting by slight centrifugation and washing using 0.5 M mannitol, the AC microcapsules were suspended in 0.15 % (w/v) sodium alginate solution for 3 minutesto generate ACA microcapsules with a solid core of alginate hydrogel. Lastly, the ACA microcapsules were suspended in a 55 mM sodium citrate solution for 5 min to liquefy the alginate hydrogel core to obtain ACA microcapsules with a liquid core.

Cell Proliferation

To investigate the long-term survival and proliferation of MSCs encapsulated in small ACA microcapsules as well as the influences of liquefying the alginate hydrogel core on cell proliferation, encapsulated cells, with or without liquefying, were seeded in cell culture inserts (BD Falcon) and used with their companion plates. The medium was renewed outside the inserts to avoid microcapsule loss every 2-3 days. WST-1 assay was performed [23] to measure the metabolic activity of the encapsulated cells. Some of the encapsulated cells were also stained with the Live/Dead® Kit for mammalian cells (Life Technologies) [2] and observed under the microscope to determine their viability every week for one month. Both 2D cultured cells and cells cultured in a 3D bulk liquid environment in ultra low attachment plates (Corning) were studied as controls. The cell seeding density was the same for all the different culture conditions and determined based on the ratio of the cell numbers/surface area of each ACA microcapsule (spherical with a diameter of 100 μm), which was 6 cells per $3.14x10^{-4}$ cm^2 in this case.

Cell Differentiation

For differentiation studies, encapsulated MSCs were cultured in regular DMEM medium for 4 weeks before use. Encapsulated MSCs that were continually maintained in regular DMEM medium without induction for differentiation was studied as control. Foradipogenic differentiation, after 3 days culture in adipogenic induction medium, the induction medium was replaced with the maintenance medium. Two days later, the medium was changed back to the adipogenic induction medium. The adipogenic induction medium was DMEM supplemented with 1 mM dexamethasone, 0.2 mM indomethacin, 0.01 mg/ml insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 10% FBS (Cyagen). The adipogenic maintenance medium was DMEM supplemented with 0.01 mg/ml insulin and 10% FBS (Cyagen). Adipogenic potential was assessed by Oil Red O staining. The cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 30 min at room temperature after 4 cycles of induction/maintenance. Then, the cells were washed with 60% isopropanol and incubated with filtered 0.3% Oil Red O (Sigma-Aldrich, St. Louis, MO) in 60% isopropanol for 30 min. After one wash in 60% isopropanol and three washes in 1x PBS, the cells were photographed. For osteogenic differentiation, the osteogenic induction medium was DMEM supplemented with 20 mM β-glycerophosphate, 50 μM ascorbate, and 10% FBS (Cyagen). The medium was changed every 3 days. After 3 weeks of induction, the cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were stained using alkaline phosphatase (ALP, Sigma) and Alizarin Red S (Cyagen) according to the manufacture's instructions. For chondrogenic differentiation, the encapsulated cells were cultured in chondrogenic induction medium (Cyagen) containing 1% (v/v) ITS+ Premix Tissue Culture Supplement, 10^{-7} M dexamethasone, 75μM ascorbate, 1 mM sodium pyruvate, 0.4 m Mproline, and 10 ng/ml transforming growth factor-beta 3 (TGF-β3). The medium was changed every 3 days. After 3 weeks, the cells were washed with 1x PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Then, the Alcian Blue staining was performed according to the manufacturer's instructions (Cyagen).

Quantitative RT-PCR (qRT-PCR) Study of Gene expression

For qRT-PCR studies, RNAs were isolated from the encapsulated cells using the method reported elsewhere [5]. Next, reverse transcription was carried out to generate complementary DNAs (cDNAs) using the iScript™cDNA synthesis kit (Bio-Rad). The reverse transcription reaction was performed using a GeneAmp PCR system (model 9700). Quantitative PCR studies were conducted with the SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real time PCR machine. Relative gene expression was calculated using the $\Delta\Delta$Ct method built in the Bio-Rad software [5]. The following genes and primers were used in this study: ADD1, 5'-GATCAAAGAGGAGCCAGTGC-3' and 5'-TAGATGGTGGCTGCTGAGTG-3'; adiponectin, 5'-GCAGAGATGGCACTCCTG-3' and 5'-CCCTTCAGCTCCTGTCATTCC-3'; αP2, 5'-ATGGGATGGAAAATCAACCA-3' and 5'-GTGGAAGTGACGCCTTTCAT-3; PPARγ2, 5'-TATGGGTGAAACTCTGGGA-3' and 5'-TGGCATCTCTGTGTCACCAT-3';Osterix, 5'-GGAGGTTTCACTCCATTCCA-3' and 5'-TAGAAGGAGCAGGGGACAGA-3'; Bone sialoprotein,5'-TGGTCTTCATTCCCCTCAGA-3' and 5'-TGAAACGGTTTCCAGTCCAG-3'; Osteocalcin, 5'-GACAAGTCCCACACAGCAGCT-3' and 5'-GGACATGAAGGCTTTGTCAGA-3'; Runx 2, 5'-GCTTGATGACTCTAAAC-

CTA-3' and 5'-AAAAAGGGCCCAGTTCTGAA -3'; Sox9, 5'-ATGAATCTCCTGGACCCCTT-3' and 5'-TTGGGGAAGGTGTTCTCCT-3'; and GAPDH (used as housekeeping gene), 5'-CTCTGGCTCAGAGGGTTTGG-3' and 5'-ACAGAAACCAGT GGGCTTTGA-3'.

Statistical analysis

All results are reported as the mean ±standard deviation (SD) of data from at least three independent runs. Student's two-tailed *t*-testassuming equal variance was calculated using Microsoft® Excel to determine statistical significance ($p < 0.05$).

RESULTS AND DISCUSSION

Typical images of encapsulated MSCs and the MSCs cultured in 3D bulk suspension at different times and the quantitative data of cell proliferation under various conditions are shown in (Figures 1,2), respectively. The cells died shortly after suspending them in ultra low attachment plates (leaving cell debris and one big cell aggregate per well finally), which was confirmed by both Live/Dead and WST-1 assay. By contrast, the MSCs survived well when suspended in the miniaturized space in the core of ACA microcapsules. For encapsulated cells, in both liquid and hydrogel cores, they maintained high viability during the entire culture period. Moreover, single cells grew into cell clusters with time, especially inthe microcapsules with a liquid core. Compared to cells cultured in Petri dishes, there was a 2-week delay in proliferation that probably was required for the cells to adjust and produce the desired matrix for them to survive and proliferate in the microcapsules. However, the cells cultured within the liquid-core microcapsules (three-dimensional (3D) cell culture) could eventually reach a higher cell density than 2D cultured cells (Figure 2A). It is worth noting that the down regulation of Runx2 and up regulation of Sox9 were observed during this study when comparing 2D-cultured cells (2D) and encapsulated cells (3D) (Figure 2B). The possible explanation is that Runx2 and Sox9 are the major transcription factors for osteogenesis and chondrogenesis, respectively, and Sox9 can inhibit the transactivation of Runx2 [24]. This suggests that the environment in theminiaturized ACA microcapsule is inductive for chondrogenesis over osteogenesis of MSCs.

During the differentiation process, the capsules remained intact and encapsulated cells showed a high viability (> 95%) during the entire period of differentiation (Figure 3). Successful differentiation of the encapsulated cells was confirmed first by immune-staining (Figure 4). For all the control groups, no staining or minor background staining could be observed, while the experimental groups showed a strong positive staining of the differentiation markers including lipid droplets stained by Oil Red Oin adipocyte-like cells formed during adipogenesis (Figure 4A-B), calcific deposition stained by Alizarin Red S (Figure 4C-D) and alkaline phosphatase secretion (Figure 4E-F) from possible osteoblasts, and sulfated proteoglycan deposits staining by Alcian blue that is indicative of the presence of functional chondrocytes (Figure 4G-H). The broken and/or swollen microcapsules werea result of staining.

Successful adipogenic and osteogenic differentiation was further confirmed by qRT-PCR studies (Figure 5). Specific transcriptional factors promoting adipogenesis including

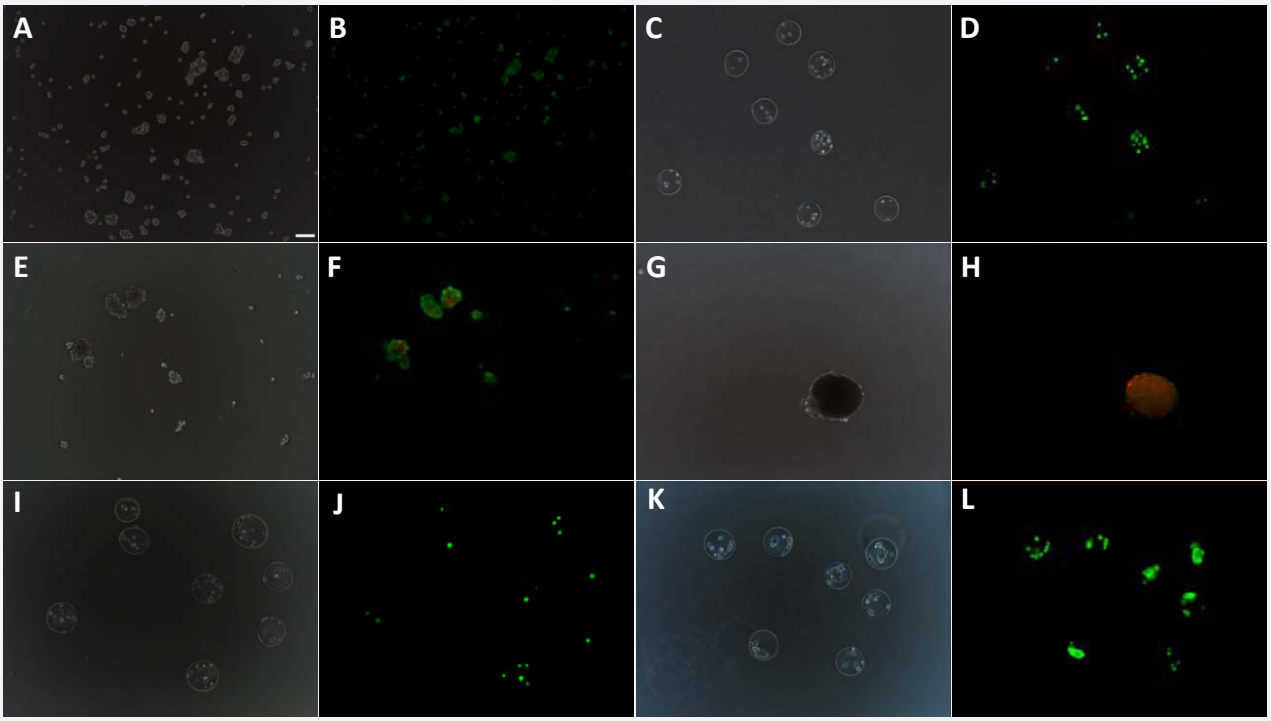

Figure 1 Typical phase contrast (A,C,E,G,I, and K) and corresponding fluorescence (B,D,F,H,J, and L) images of C3H10T1/2 cells cultured under different conditions at different times: cells cultured in bulk suspension (A and B) at day 0 and capsulated cells immediately after coating (C and D); cell cultured in suspension at day 2 (E and F) and day 7 (G and H); and cell encapsulated in ACA microcapsule with a hydrogel (I and J) and liquid (K and L) core, respectively, after cultured in regular DEME for 1 month. Scale bar: 100 μm.

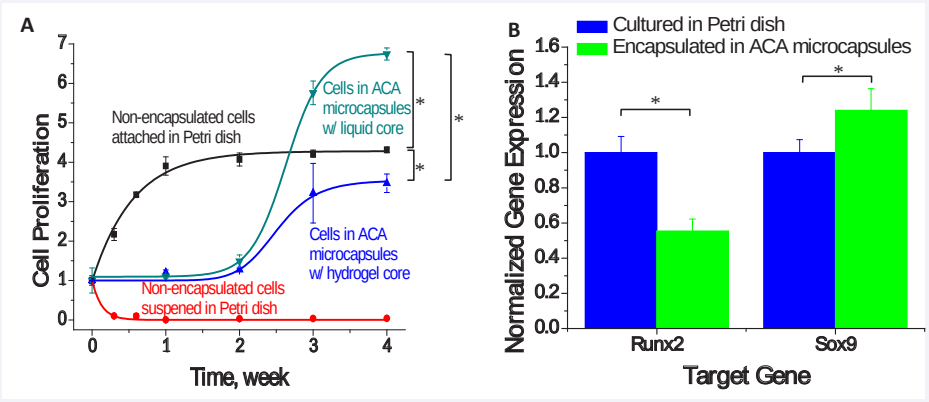

Figure 2 Proliferation of C3H10T1/2 cells cultured under four different conditions for 4 weeks(A)and Expression of Runx2 (osteoblast) and Sox9 (chondrocyte) genes in C3H10T1/2 cells cultured in Petri dish (2D) and the miniaturized 3D liquid core of ACA microcapsules (B). *denotes significant difference (p<0.05).

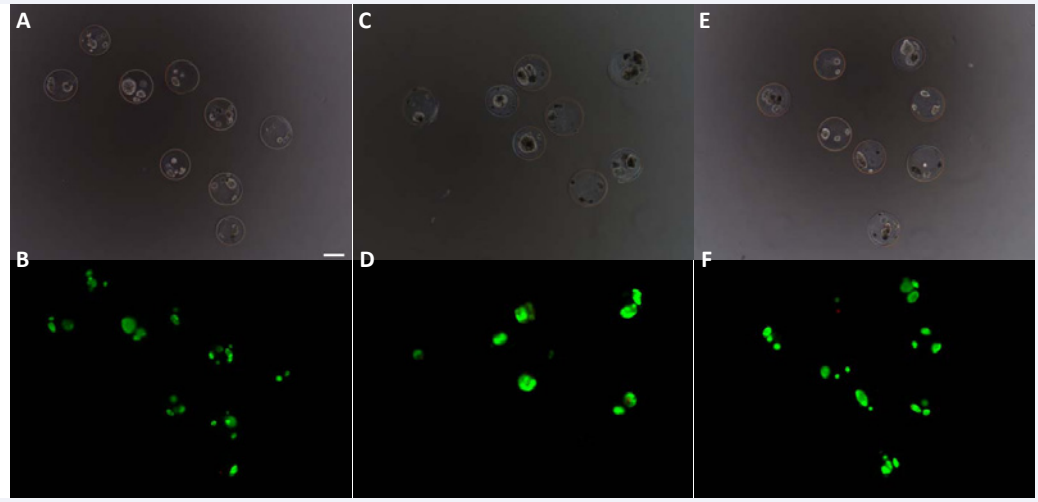

Figure 3 Typical phase contrast (A,C, and E) and corresponding fluorescence (B,D, and F) images of encapsulated C3H10T1/2 cells cultured under different conditions: cells cultured in DMEM for 7 weeks (A and B); cells cultured in DMEM for 4 weeks and then adipogenic differentiation medium for 3 weeks (C and D); and cells cultured in DMEM for 4 weeks and then osteogenic differentiation medium for 3 weeks (E and F). Scale bar: 100 μm.

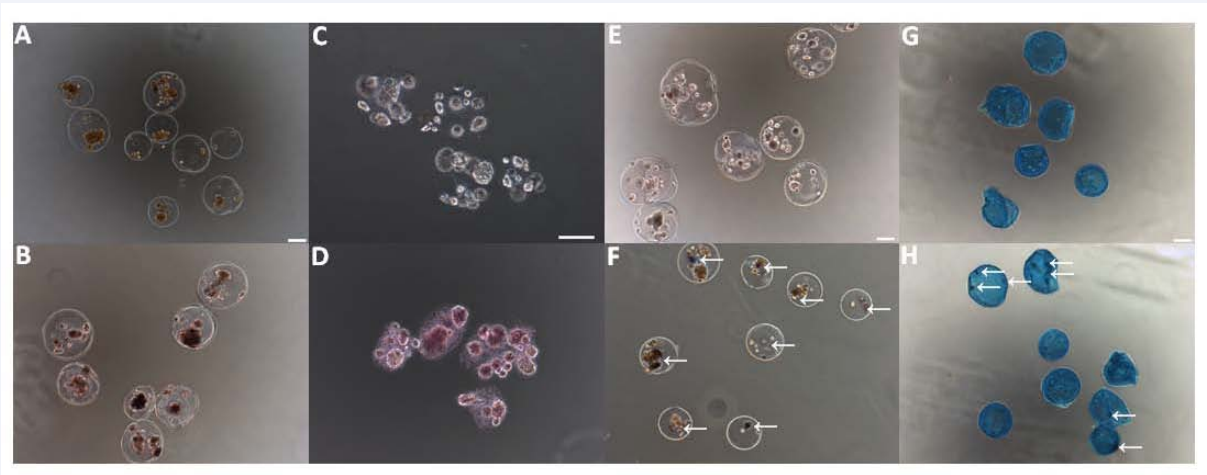

Figure 4 Typical phase contrast images of non-differentiated (A,C, and E) and differentiated (B,D, and F) encapsulated C3H10T1/2 cells (within or released from ACA microcapsules): Oil Red O staining (red; A and B), Alizarin Red S staining (red; C and D), ALP staining (blue; E and F), and AlcianBlue staining (blue; G and H). Arrows indicate positively stained cells. Scale bar: 100 μm.

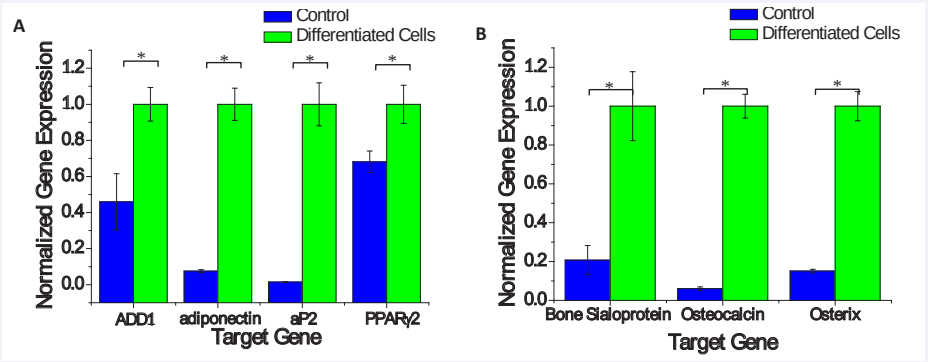

Figure 5 Changes in gene expression of the encapsulated C3H10T1/2 cells without and withadipogenic (A) and osteogenic (B) differentiation.The cells without differentiation were studied in parallel for control. *denotes significant difference (p<0.05).

adipocyte determination and differentiation factor 1 (ADD1) and peroxisome proliferators activated receptor-γ (PPAR-γ), adipose-specific genes (adiponectin), and adipocyte-specific fatty acid-binding protein (aP2) genes, were studied for adipogenesis. At the same time, osteoblast-specific transcription factor (osterix) and bone extracellular matrix genes (bone sialoprotein and osteocalcin) were investigated for osteogenic differentiation. The expression of all these important differentiation markers was significantly higher in the cells after adipogenic or osteogenic differentiation, compared to the control (i.e., non-differentiated) cells (Figure 5).

CONCLUSION

C3H10T1/2 cells survived and proliferated well during the 4-week-long period. Encapsulated cells started to proliferate rapidly, growing from single cells into cell aggregates/clusters after 2 weeks. The proliferation could be improved by incorporating a natural ECM, such as collagen into the microcapsule core [7,8,25,26]. In addition, the encapsulated C3H10T1/2 cells were successfully induced to undergo adipogenic, osteogenic, and chondrogenic differentiation. Overall, the small ACA microcapsule is a promising system for MSC encapsulation and directed differentiation for cell-based therapies and tissue engineering.

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