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Original article

Matrigel Enhances *in vitro* Bone Differentiation of Human Marrow-derived Mesenchymal Stem Cells

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Abstract

Objective(s)
The use of co-culture cells as well as extra cellular matrix are among those strategies that have been employed to direct mesenchymal stem cell (MSC) bone differentiation in culture. In this regard, there is no study considering the effects of Matrigel on mesenchymal stem cell (MSC) *in vitro* bone differentiation. This was the subject of the present study.
Materials and Methods
Human passaged-3 MSCs isolated from the marrow aspirates were seeded on either Matrigel or conventional polystyrene plastic surfaces (as control) for 10 days. To compare the cell proliferation in two cultures, the cell numbers were determined during the cultivation period. For bone differentiation, the confluent cultures from either group were provided with osteogenic medium and incubated for 21 days during which the alkaline phosphates (ALP) activity, culture mineralization and the expression of some bone-related genes were quantified and statistically compared.
Results
MTT assay indicated that Matrigel-cultivated cells underwent statistically less proliferation than polystyrene-cultivated cells ($P<0.05$). Regarding the osteogenic differentiation, ALP activity was significantly high in Matrigel versus plastic cultures. Calcium deposition in Matrigel cultures tended to be significantly extensive compared with that of control cultures (2.533±0.017 versus 0.607±0.09 mM). Furthermore, according to the semi-quantitative RT-PCR analysis, compared with polystyrene plastic surface, Matrigel seemed to provide a microenvironment in which human MSC expressed osteocalcin and collagen I genes in a significantly higher level.
Conclusion
Collectively it seems that Matrigel could be considered as an appropriate matrix for MSC osteogenic differentiation.

Keywords: Cell proliferation, Matrigel, Mesenchymal progenitor cells, Osteogenesis

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Introduction

Basement membrane (BM) is the first extra cellular matrix that is produced in developing embryos. Since stem cells constituting developing embryos are first come in contact with BM macromolecules, investigators are believed that BM could have a profound impact on stem cell differentiation (1-2).

In adults, BM is located either under the basal surface of epithelial cells or around of muscle, adipose and schwann cells (3). Structural support of the cells, storing growth factors and cytokines, participation in signal transduction and maintaining cell differentiated phenotype have been considered as BM functions in tissue (4-6). It has been shown that the impairment of cell attachment on ECM may result in apoptotic death in the cell population (7-8).

Matrigel is a solubilized tissue basement membrane matrix rich in extracellular matrix proteins that was originally isolated from the Engelbreth-Holm-Swarm (EHS) mouse tumor (9). It is mainly composed of laminin. The other constituents include collagen IV, proteoglycan, heparin sulfate, entactin, nidogen, and several growth factors which naturally exist in EHS (10). Some studies have shown that Matrigel as complex extra cellular matrix is able to promote cellular differentiation *in vitro*. In this regard, sertoli cells have been shown to grow as columnar cells on Matrigel culture and produce glandular structures with cavities (11). Epithelial cells from human oviductal and uterine tissue have assumed polarized morphology on Matrigel surface (12-13). Furthermore, it has been demonstrated that immortalized and umbilical endothelial cells are able to produce vessel-like structures in Matrigel culture (14-15). Cells cultivated in Matrigel have been reported to even become functional following differentiation in Matrigel culture. For instance, epithelial cells of salivary gland plated in Matrigel are able to secret amylase into the culture medium (16).

Differentiating effects of extra cellular matrix components have also been reported in stem cell cultures. In a study by Salasznyk *et al* it has been indicated that the use of either vitronectin or collagen I can promote MSC bone differentiation in culture (17). The use of laminin-5 has been shown to induce osteogenic differentiation of MSCs (18). According to former investigations, the use of extra cellular matrix may also promote MSC chondrogenic differentiation. Bradharm *et al* have reported that the amount of chondrocyte production in MSCs Matrigel culture was four folds more than that of micro-mass culture system (19). Complex extra cellular matrix has also been reported to have an impact on chondrogenic differentiation of the embryonic stem cells in culture (20). To our knowledge there is no report on effects of Matrigel as a matrix for marrow-derived MSCs culture. MSCs are multipotent cells having the potential to differentiate into skeletal cell lineages when being provided with appropriate micro-environment (21). These cells are considered to be an important candidate for reconstructing bone and cartilage defects. The factors involving in MSCs differentiation are not recognized well (22). MSCs differentiation into various lineages is basically dependent on the presence of specific inducers in culture medium. The aim of this study was to investigate the effects of Matrigel on MSCs culture. For this purpose, culture-expanded human marrow-derived MSCs were used to establish a monolayer culture on either Matrigel or conventional polystyrene plastic surfaces. Under these conditions, the proliferation and bone differentiation of the cells were quantitatively determined and statistically compared to each other.

Materials and Methods

Bone marrow obtaining

The use of the human bone marrow for research was approved by the Ethics Committee of Royan Institute (Tehran, Iran). Bone marrow was obtained from the patients who were volunteer for stem cell transplantation after myocardial infarction. In this study the bone marrows having been left after immunodepletion for AC133 positive cells (which were autologously transplanted into the patient heart defect) were utilized to harvest mesenchymal stem cells.

MSC isolation

In order to isolate mesenchymal stem cells, bone marrow was mixed with phosphate buffer solution in equal ratio; the mixture was loaded on Ficoll (inno train) in 3:7 ratios and centrifuged at 1100 RPM for 5 min. The fraction containing mononuclear cells was removed using pipette and the cells were suspended in 10^6 cells/ml in DMEM (Dulbecco's Modified Eagle Medium, Gibco, UK) supplemented with 15% Fetal Bovine Serum (FBS, Gibco), 100 IU/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma, Germany) and plated. The cultures were incubated at 37 °C in humidified atmosphere of 5% CO_2. The first medium replacement was done on day 5 of culture and the subsequent changes of medium were performed every 3 days. The first passages were performed about two weeks after culture initiation when 70% of culture surface was covered with the cells. Several further subcultures were performed before the sufficient cells being provided for the following experiments.

Preparation of Matrigel-coated culture dishes

Matrigel (Sigma) was diluted to 0.34 mg/ml in DMEM without FBS. Six well culture plates were then added with about 1 ml diluted Matrigel and incubated at 37 °C for 4 hr at the end of which the excess Matrigel was removed from the wells.

MSCs proliferation culture

MSCs from passaged-3 cultures were plated at 1×10^4 cells per well of 6-well plates coated with Matrigel. The same number of the cells was plated in 6-well plates without Matrigel coating (control group). The culture was provided with DMEM supplemented with 15% FBS and antibiotics for 10 days during which the cell proliferation was quantitatively determined by MTT assay and compared to each other.

MTT assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazulium bromide] (Sigma, USA) mitochondrial reaction was used to determine the cells number in different time points including days 3, 7 and 10 of culture period. This assay based on the ability of live cells to reduce a tetrazulium-based compound, MTT, to a purplish formazan product. Briefly, the cultures were washed with PBS, added with the solution containing 5:1 ratio of media and MTT solution (5 mg/ml in PBS), and incubated for 2 hr at 37 ºC. Medium and MTT solutions were removed and 0.5 ml of extraction solution (dimethylsulphoxide: DMSO) was added to dissolve the foramazone precipitate. The absorbance of the supernatant was read with a microplate reader (BioTek ELX×800) at 540 nm. Cells number was determined through a standard curve that was established by using a known number of cells counted by a coulter counter.

MSC osteogenic culture

To create bone differentiation culture, 10^5 passaged-3 cells were seeded on either Matrigel-coated or uncoated plastic surfaces of 6-well polystyrene culture plate. Upon confluency, the proliferation medium (DMEM containing FBS and antibiotics) was replaced by osteogenic medium (DMEM supplemented by 50 µg/ml ascorbic 2 phosphate, 10^{-8} mM dexamethsone and 10 mM ß glycerol phosphate) and the cultures were incubated for additional 21 days at 37 °C and 5% CO_2. Confluent culture of MSCs on polysterene plastic surfaces and the culture of MSCs on Matrigel-coated plate without osteogenic inducers were taken as the control. To compare the bone differentiation of the MSCs in cultures, alkaline phosphatase activity (ALP), culture mineralization and the relative bone-specific gene expression including osteocalcin and collagen I were determined for each culture at various time points including days 7, 14 and 21 of culture period and the mean values were statistically compared to each other.

Alkaline phosphates (ALP) activity

To investigate the ALP activity of the osteogenic cultures, the cells in culture were washed with PBS, homogenized with 1 ml Tris buffer (pH 7.4, Sigma) and sonicated. The cell lysate (0.1 ml) was mixed with 0.5 ml

p-nitrophenol phosphate (PNP) substrate solution (Sigma) diluted in 0.5 ml ALP buffer solution (Sigma) and incubated at 37 °C for 15 min. The solution was then added with 10 ml 0.05 N NaOH and its optical absorbance was recorded at 405 nm by spectrophotometer. Total protein content was determined using Bradford method (Bradford 1976) in aliquots of same samples and calculated according to a series of gamma-globulin standards.

Culture mineralization

The intensity of alizarin red staining for osteogenic cultures was quantified and compared using osteogenesis quantification kit (Chemicon, USA). This analysis was performed by determining OD_{405} values of a set of known alizarin red concentrations and comparing these values to those obtained from the osteogenic cultures. The procedure was performed according to the manufacturer's instruction. In brief, the cultures were fixed in 10% formaldehyde for 15 min and followed by staining with alizarin red solution for 10 min. The cultures were washed with distilled water and were added with 10% acetic acid which resulted in dissolving of red matrix precipitate. The optical density of solution was read at 405 nm with a microplate reader and compared to those of known alizarin red concentrations being provided by the kit supplier.

Semi quantitative RT-PCR

Total RNA was isolated from the cells in culture using the Nucleospin RNAII kit (Macherey-Nagel, Germany) according to the manufacturer's specifications. The RNA samples were digested with DNase I (EN0521; Fermentas) to remove contaminating genomic DNA, and then quantified spectrophotometrically at 260 nm. All RNA isolates had an OD260:OD 280 between 1.8 and 2.0, indicating clean RNA isolates. A two-step semi-quantitative RT-PCR method was used to measure the specific gene expression during osteogenic differentiation of mesenchymal stem cells. Standard RT was performed using the RevertAid TM H minus First Strand cDNA Synthesis Kit (K1622; Ferments, Germany) and random hexamer primer was used as primer in the first step of cDNA synthesis. Relative RT-PCR was performed to measure gene expression of human osteocalcin and COL I. Primer sequences and optimal PCR annealing temperatures (AT) are listed in Table 1. Polymerase chain reactions were performed on a PTC-200 PCR machine (MJ Research Inc, MA, USA) using 2 µl cDNA, 1×PCR buffer (AMS^{TM}; CinnaGen Co., Tehran, Iran), 200 µM dNTPs, 0.5 µM of each of forward and reverse primers and 1U Taq DNA polymerase (Fermentas, MD, USA). PCR reactions were performed on a Mastercycler gradient machine (Eppendorf, Germany). Amplification conditions were consisted of initial denaturation, 94 °C for 5 min followed by 35 cycles (25 cycles for GAPDH) of denaturation at 94 °C for 45 sec, annealing for 45 sec, extension at 72 °C for 30 sec, and a final polymerization at 72 °C for 10 min. Each PCR was performed under linear conditions with GAPDH used as an internal standard. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidium bromide (0.5 mg/ml) and photographed on a UV

Table 1. Primers used in semi quantitative RT-PCR analysis.

GENE	Sequences 5'---3'	Expected fragment size (bp)	Annealing temperature (°C)	GenBank accession number
BGLAP (osteocalcin)	Forward: GGCAGCGAGGTAGTGAAGAG Reverse: CAGCAGAGCGACACCCTAGAC	195	63	NM_199173
COL1A1 (collagen type I)	Forward: GTGGTGACAAGGGTGAGACAG Reverse: CAACAGGACCAGCATCACCAG	255	62	NM_000088

transilluminator (uvidoc, UK). The gel images were digitally captured with a CCD camera and analyzed using the UVI band map program (Uvitec, Cambridge, UK). For semi-quantitative determination of mRNA levels of the candidate genes, transcript levels were normalized to the corresponding GAPDH. Data were analyzed by SPSS 13.0 for windows.

Statistical analysis

Mean values±SD (standard deviation) were calculated for the data obtained from the MTT assay, mineralization quantification, ALP assay and RT-PCR analysis. The data were analyzed with T-test using SPSS software version 13. A *P*- value <0.05 was considered to be significant.

Results

Cell culture

Although most blood cells had already been removed from the bone marrow thanks to ficoll gradient centrifugation, some hematopoietic cells were still present in primary cultures. These cells were gradually eliminated as the primary cultures passaged. The cultures appeared to be purified after three successive subcultures. Matrigel-cultured cells (Figure 1A) tended to be somewhat thinner and elongated than plastic-cultivated cells (Figure 1B).

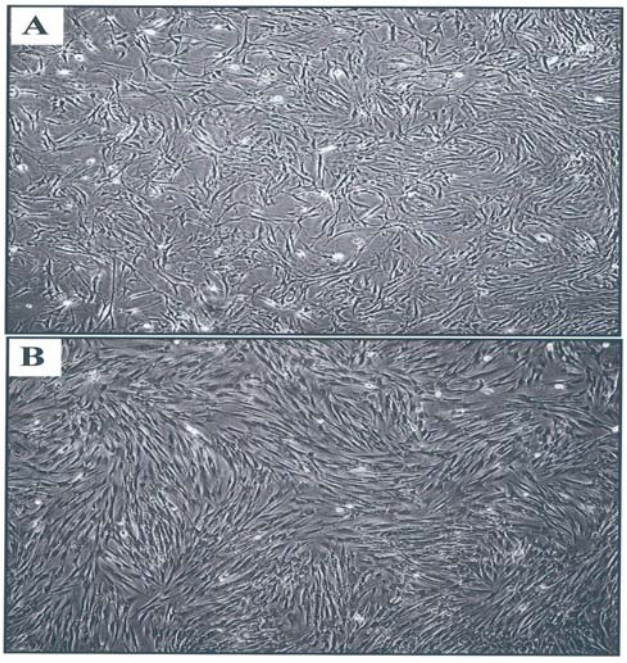

Figure 1. Representative photomicrograph of passaged-3 human mesenchymal stem cells on Matrigel surface (A) and conventional polystyrene plastic surface (B). Magnification: ×40

Cell proliferation

Polystyrene-cultivated MSCs possessed more rapid proliferation rate than their counterparts on Matrigel surface during the all studied time points of cultivation period. The value of cell number on day 10 for the culture without Matrigel was 99.42±5.99 (Figure 2A). This was statistically (P<0.05) more than that of Matrigel-cultivated cells (74.28±6.7).

Bone differentiation

Alkaline phosphatase activity of the cells

Alkaline phosphatase as a cell surface glycoprotein has a role in matrix mineralization process. ALP activity of both culture groups was significantly increased as the culture time advanced. There was no significant difference between two cultures on day 7, but on day 14 and 21 ALP activity tended to be higher (three times) in Matrigel- versus plastic-cultivated cell cultures (P<0.05) (Figure 2B).

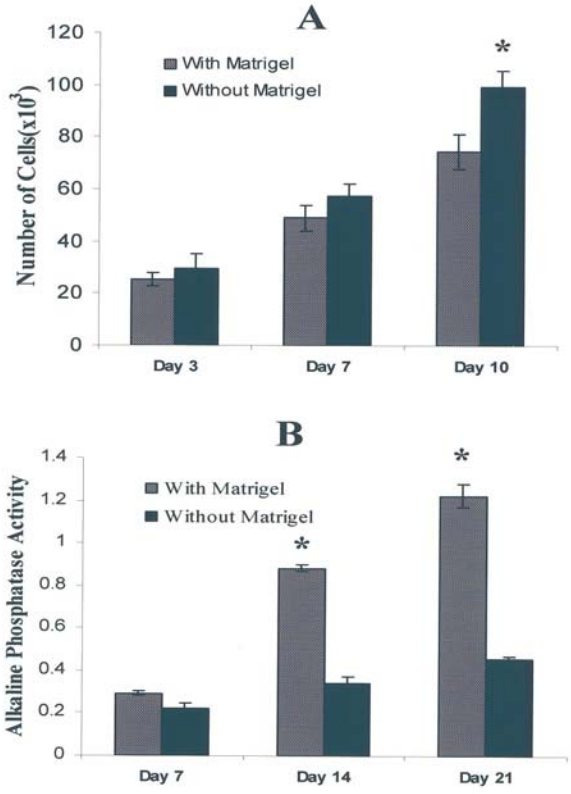

Figure 2. A) A graph showing the significantly high proliferation of human mesenchymal stem cell on polystyrene plastic versus Matrigel surface. B) Human passaged-3 mesenchymal stem cells cultivated on Matrigel surfaces under osteogenic conditions indicated a significantly higher alkaline phosphatase activity compared to that of conventionally plastic-cultivated cells. * indicates the statistically significant difference (P<0.05).

Culture mineralization

Matrix mineralization is one of those characteristics events during bone development. In the present study, this was quantified by the alizarin red staining method (Figure 3A-B). According to our findings, the mineralization tended to increase in the cultures as the culture time progressed (Figure 3C). Similarly, there was no significant difference between two cultures on day 7, but the differences on day 14 and 21 appeared to be statistically significant. On all studied time points, calcium precipitation in Matrigel culture was significantly more than polystyrene cultures (P<0.05). The value for Matrigel cultures was 2.533±0.017 mM on day 21 compared to the value of 0.607±0.09 mM for polystyrene cultures at the same time point.

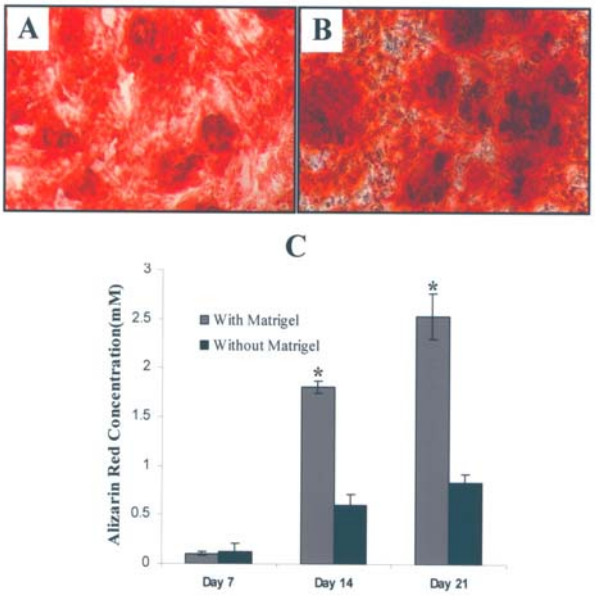

Figure 3. Measurment of culture mineralization in osteogenic cultures of human mesenchymal stem cells. A) Alizarin red staining of osteogenic culture on conventional polysterene plastic surface. B) Alizarin red staining of osteogenic culture on Matrigel surface. C) A graph indicating the comparative alizarin red concentrations (as an index of the culture mineralization) in osteogenic cultures. * indicates the statistically significant difference (P<0.05).

Osteogenic genes expression

According to the semi-quantitative RT-PCR analysis, the presence of Matrigel in the osteogenic cultures resulted in significantly increased expression of osteogenic marker gene including collagen I and osteocalcin at the all studied time points (Figure 4A-B). The differences were statistically significant (P<0.05).

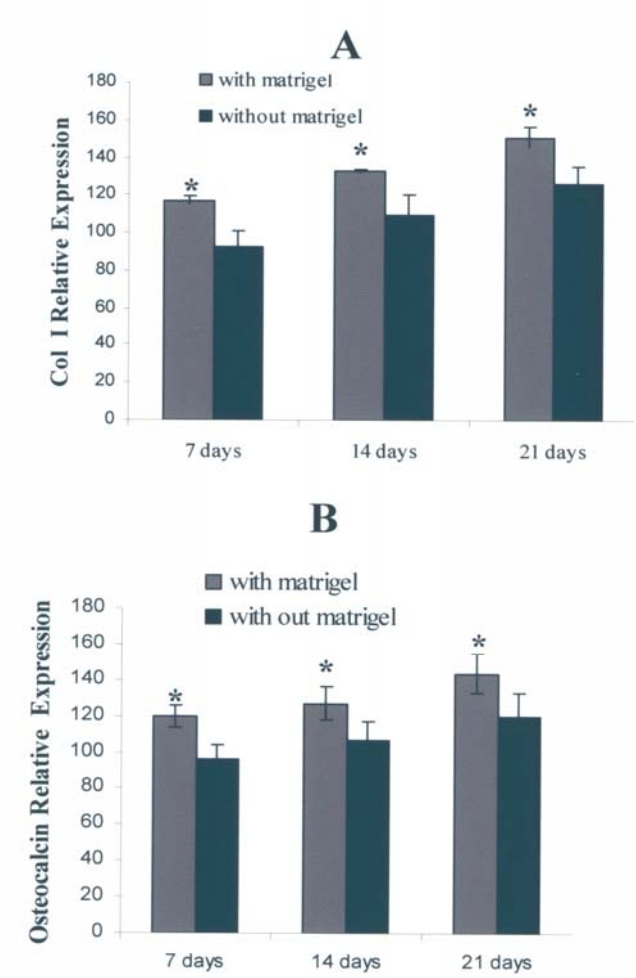

Figure 4. Relative osteogenic gene expression in mesenchymal stem cell culture on conventional polystyrene plastic surface and Matrigel surface. The expression of collagen I (A) and osteocalcin (B) in Matrigel culture was much similar to that in intact osteocytes. *indicates the statistically significant difference (P<0.05).

Discussion

In the present study the effects of Matrigel on proliferation and bone differentiation of human marrow-derived MSCs was investigated. To date, there is no report on the subject of Matrigel effects on MSCs *in vitro* proliferation and bone differentiation. The topics of MSCs osteo differentiation would be of great clinical interest. In general, there could be two strategies with respect to the use of MSCs-mediated tissue regeneration. One route would be to use the cells as undifferentiated state, allowing them to be differentiated in *in vivo* micro-environment. The disadvantage of this approach is that unwanted cell differentiation other than the desired cells may occur in transplantation site.

For instance, if the cells are to regenerate bone tissue, other cells may produce in the site by unwanted differentiation (23-24). Therefore, the alternative approach is to fully differentiate the cells down the desired cells prior to their transplantation. Such strategy could indeed guarantee the administration of the intrinsic differentiated cells into the repair site. Considering such strategy, the importance of the MSC in *in vitro* differentiation down bone tissue would be clarified. According to the present study, the use of Matrigel in human marrow-derived MSC osteogenic cultures can significantly enhances their bone differentiation. Study like this would help to produce fully differentiated cells for application in regenerative medicine.

While regenerating the bone large defects requires considerable number of stem cells, the number of MSCs have been reported to be scarce in tissues including bone marrow (22). For this reason, MSCs in *in vitro* propagation is considered as an inevitable step prior to their application in cell-based treatment of tissue defects. In the present study, the subject of Matrigel effects on MSCs proliferation was considered as well. According to our results, it was appeared that Matrigel possesses inhibitory effects on MSC *in vitro* proliferation. Therefore, Matrigel should be excluded from MSCs culture when their expansion is being desired. On the other words, Matrigel can be included /accompanied MSCs when MSCs proliferation is no longer required. This is the case when MSCs are to be transplanted in defect site where further proliferation (growth) of the cells is unwanted.

The data by the present study was indicated that in general Matrigel enhances MSCs bone differentiation and decreases their *in vitro* proliferation. These results are in agreements with basement membrane function in *in vivo* conditions where it was believed that one important function of basement membrane is to maintain the cell differentiated phenotype. Enhancement of bone differentiation of MSCs that was observed in this study could be attributable to Matrigel specific components. Matrigel is indeed an artificial basement membrane consisting of several macromolecules. Of major components of Matrigel are its collagen (25) and laminin (18) contents which have been shown to have differentiating effects on the cells while inhibiting their proliferation.

Conclusion

Collectively, it seems that Matrigel enhances human marrow-derived MSCs bone differentiation in culture and inhibits their proliferation. Given these data, it seems that including Matrigel to tissue construct composed of MSCs that are considered to be transplanted into bone defects presents two interesting benefits; first it enhances bone differentiation and second it prevents cell proliferation reducing the possibility of abnormal growth.

Acknowledgment

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