Established Preblastocyst- and Blastocyst-Derived ES Cell Lines Have Highly Similar Gene Expression Profiles, Despite Their Differing Requirements for Derivation Culture Conditions

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Abstract

The efficiency of embryonic stem (ES) cell derivation relies on an optimized culture medium and techniques for treating preimplantation stage embryos. Recently, ES cell derivation from the preblastocyst developmental stage was reported by removing the *zona pellucida* from embryos of the most efficient strain for ES cell derivation (129Sv) during early preimplantation. Here, we showed that ES cells can be efficiently derived and maintained in a modified medium (MEM α), from preblastocysts of a low-efficiency mouse strain (a hybrid consisting of 50% B6, 25% CBA, and 25% DBA). Preblastocyst-derived ES cell lines were normal in terms of pluripotency-related protein expression, and chromosome number. Also, preblastocyst-derived ES cell lines from various culture conditions showed pluripotency *in vivo* through teratoma analysis. Interestingly, ES cell lines produced from preblastocysts and blastocysts, regardless of the derivation culture conditions, are nearly indistinguishable by their global gene expression profiles.

Introduction

ES CELL DERIVATION from early preimplantation stage embryos is based on the idea that cells from this earlier time point may exhibit a wider developmental potential than those of inner cell mass (ICM) or epiblast origin. In general, the early preimplantation embryos prior to blastocyst development have been evaluated by dissociating them into individual blastomeres for ES cell derivation, but this method produces ES cell lines at very low efficiencies (Chung et al., 2006; Delhaise et al., 1996; Wakayama et al., 2007). Recently, a new technique involving removal of the *zona pellucida* has been introduced, with subsequent blocking of cavitation in the embryo (Tesar, 2005). Tesar showed that whole denuded (*zona*-free) embryos from early preimplantation stages (zygote, four-, eight-cell, and morula stage) could develop, attach, and spread out to form colony-like structures in culture, after they reached a morula-like stage (preblastocyst) on

feeder layers without cavitation. Tesar demonstrated that putative ES cell lines could be obtained in this way with high efficiency from 129S2/SvHsd embryos using standard culture medium (Knockout-DMEM, KDMEM) for derivation and maintenance, which are typically used for ES cell derivation (Tesar, 2005). It is currently not known whether this method can be successfully applied to nontraditional mouse strains, nor is it well understood what is different between preblastocysts and *zona*-intact embryos (blastocysts), in terms of development and characterization of ES cells derived from these two different developmental stages.

In preimplantation embryos, there are dramatic changes in pluripotency-related gene (*Oct4*) expression and glucose consumption as embryos develop from the morula to the blastocyst stage. *Oct4* transcripts are detectable at a basal level from the one-cell to eight-cell stages, and the expression levels of *Oct4* increased significantly at the morula stage in *in vitro* cultured embryos (Li et al., 2005). Between the

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morula-blastocyst stage and implantation in mouse and human embryos, amounts of glucose uptake are increased (Leese, 1991; Martin and Leese, 1995). In a previous study, we reported that modified MEM α containing high glucose improves the efficiency of ES cell derivation, compared with low glucose MEM α and Knockout (KDMEM) by supporting Oct4 expression (Kim et al., 2009).

In the present study, we first compared the development of the preblastocysts, and zona-intact embryos on feeders. Second, we used Oct4-GFP expression to evaluate three distinct medium formulations in an effort to improve ES cell derivation efficiency from preblastocysts in a nonstandard mouse strain (50% B6, 25% CBA, and 25% DBA), which is the same strain with our previous study for ES cell derivation from blastocyst (Kim et al. 2009). Third, gene expression profiling was used to compare ES cells derived from preblastocysts and blastocysts in different culture conditions.

Materials and Methods

Preblastocyst preparation and ES cell derivation

Male mice (CBA/CaJ X C57BL/6J; Jackson Laboratories, West Grove, PA) which have an Oct4-GFP transgene, were mated with female BDF1 mice (C57BL/6 $\times$ DBA/2)F1 (Jackson Laboratories) post-PMSG and HCG injection. Two-cell embryos were collected by flushing the uterine horns at 35 h post-HCG injection. Zona *pellucida* were removed with brief exposure to acidic Tyrode's Solution (Millipore, Bedford, MA) for zona-free embryo preparation. Zona-free and zona-intact embryos were cultured individually in a 96-well dish for 96 h on primary mouse embryo fibroblasts (feeders, Millipore) treated with mitomycin C (10 μ g/mL, Sigma-Aldrich, St. Louis, MO) in KSOM. Development of the embryos was checked at 24, 72, and 96 h. Preblastocysts, which were attached to feeders without cavitation, were detached and treated with 0.05% Trypsin-EDTA for 1 min and dissociated with a glass pipette into small pieces and transferred to new feeders in each of three different culture media (described below). Oct4 expression was evaluated during ES cell derivation by detecting GFP.

Effects of culture medium and epigenetic modification, as evaluated by Oct4-GFP expression

Three culture media were evaluated for ES cell derivation from preblastocyst; (1) LG-MEM α (1000 mg/L of glucose; Invitrogen, Carlsbad, CA) supplemented with 1 $\times$ β -mercaptoethanol (Millipore), 1000 U/mL of leukemia inhibitory factor (LIF; Millipore), 15% fetal bovine serum (FBS) (Gibco, Grand Island, NY), and penicillin-streptomycin (Gibco); (2) modified MEM α (HG-MEM α ; same as the MEM α above except with 4500 mg/L of glucose; Invitrogen); and (3) KDMEM (4500 mg/L of glucose; Invitrogen) with 1 $\times$ β -mercaptoethanol, 1000 U/mL of LIF, penicillin-streptomycin, 2 mM glutamine (Sigma-Aldrich), and 1 $\times$ nonessential amino acid solution (Sigma-Aldrich). All cells were incubated at 37°C in 5% CO₂ atmospheres.

Immunostaining

Mouse monoclonal antistage-specific embryonic antigen-1 (SSEA-1; Millipore) was diluted 1:50 and incubated with cells

for 1.5 h at room temperature. Alexa-fluor 594 goat anti-mouse IgM (Invitrogen, red color) was diluted to 1:500 and incubated with cells for 1.5 h at room temperature. 4',6'-Diamidino-2-phenylindole hydrochloride (DAPI, Sigma; 100 ng/mL) was incubated with cells to visualize DNA. Oct4 expression was detected by GFP without staining under fluorescent microscopy.

Teratoma study

ES cell lines derived from preblastocysts and blastocysts maintained in three different media were dissociated by 0.05% Trypsin-EDTA at passage 12. Total 2.0 $\times$ 10⁶ cells were injected subcutaneously with culture medium using a 25-G needle into three severe combined immunodeficiency disease (SCID) mice per ES cell line (Charles River Laboratories, Raleigh, NC). Teratoma formation was examined for 4 weeks until tumor growth necessitated the sacrifice of the animal. ES cell-derived tumors were fixed and tissue sections were stained with hematoxylin-eosin to visualize the general morphology.

Microarray

ES cells were maintained in the same culture medium as that for derivation and then cultured on a gelatin-coated dish without feeders for one passage before extraction of total RNA. Microarray analysis was performed as previously described (Park et al., 2011). Briefly, each cell line total RNA was reverse transcribed, labeled, and hybridized in dye-swapped pairs with labeled Universal Mouse Reference cDNA to 38K Mouse Exonic Evidence-Based Oligonucleotide (MEEBO) arrays. Each slide contained 38,467 individual 70-mer oligonucleotide probe features, interrogating nearly 25,000 mouse genes, and each experimental class contained four replicates, consisting of two dye-swap pairs. Data were analyzed in GeneSpring 7.3 (Agilent Technologies, Palo Alto, CA) as previously described (Abbondanzo et al., 1993). Multiple comparison strategies were used to identify DE gene sets. First, all possible pairwise comparisons were examined using a parametric *t*-test with variances not assumed equal, a Benjamini-Hochberg false discovery rate (FDR) threshold of 0.05, and a fold-change minimum of 2.0. Second, a one-way ANOVA (Welch, parametric with variances not assumed equal) was performed (FDR $\leq$ 0.05 and fold-change $\geq$ 2.0). Third, to identify genes that are differentially expressed according to the experimental parameters (preblastocysts vs. blastocyst) and the derivation culture conditions (KDMEM, LGMEM α , or HGMEM α), a two-way ANOVA analysis (parametric, variances assumed equal) was performed (FDR $\leq$ 0.05 and fold-change $\geq$ 2.0). Gene lists were examined and characterized manually, and loaded into MetaCore software (GeneGo, Joseph, MO) for pathway/network analyses. Hierarchical clustering analysis using the similarities between the various experimental classes, as well as between expression patterns of the individual genes, was performed in GeneSpring 7.3 using the DE gene sets identified by ANOVA analysis.

Statistical analysis

ES cell line derivation efficiency rates were analyzed using a one-sided chi-square test ($p < 0.05$).

TABLE 1. ZONA-FREE AND -INTACT EMBRYO DEVELOPMENT ON FEEDERS UP TO 96 HOURS

Starting material	No.	24 h			72 h					96 h (%)			
		<6	8	M	<8	M	B	PB	D	M	B	PB	D
Zona-Free	260	87	163	10	0	36	48	175	1	0	62 (23.8)	194 (74.6)	4 (1.6)
Zona-intact	235	99 ^a	124 ^a	12	0	9 ^a	221 ^a	0 ^a	5 ^a	0	225 (95.7) ^a	0 ^a	10 (4.3) ^a

Zona-free, two-cell embryos with zona pellucida removed; Zona-intact, two-cell embryos with zona pellucida intact; <6, embryos at six-cell stage or earlier; 8, eight-cell stage embryos; M, morula-stage embryos; B, blastocyst-stage embryos; PB, preblastocysts (colony-like attachment); D, degenerated embryos.

^aDevelopment between zona-free and zona-intact embryo, one-sided chi-square-test ($p < 0.05$).

Results

Development of preblastocysts by zona pellucida removal

Zona-free and zona-intact embryos were placed into individual wells of a 96-well plate on feeders, and their development was compared for 96 h (Table 1 and Fig. 1). Zona-free embryos developed to eight-cell or morula stages significantly faster than zona-intact embryos at 24 h postcultivation (66.5 and 57.8%, respectively). At 72 h cultivation, most zona-free embryos at the morula stage had begun to attach to the feeder layer, without visible cavitation (67.3%, preblastocyst), but a minority (18.5%, blastocysts) did undergo some degree of cavitation. Overall, 13.8% of zona-free embryos did not proceed in development (Fig. 1B), remaining at the morula stage. In contrast, most zona-intact embryos (94%) had developed into blastocysts at 72 h. A total of 74.6% of

zona-free embryos had developed into preblastocysts after 96 h culture on feeders (Table 1 and Fig. 1C), whereas only 23.8% developed to blastocyst. We observed that 100% of preblastocysts were expressing strong Oct4-GFP signals in their centers (Fig. 1C') and we selected well-formed, Oct4-GFP-expressing preblastocysts for ES cell derivation. Again, a higher percentage (95.7%) of the zona-intact embryos had become blastocysts by the 96-h time point.

Oct4-GFP expression persistence and derivation efficiency of ES cell lines from preblastocysts in different culture conditions

Three different culture media were evaluated for ES cell derivation from preblastocysts by monitoring of Oct4-GFP expressing cells following passage. Efficiency of ES cell derivation (Table 2) was also determined. At 2 h after

FIG. 1. Process of preblastocyst preparation and ES cell derivation. (A) Eight-cell denuded embryo at 24 h postcultivation. (B) Denuded morula at 72 h. Postcultivation. (C, C') Oct4-GFP expression in preblastocyst at 96 h postcultivation on feeders. (D, D') Dissociated cell mass with Oct4-GFP expression at 2 h after first passage in LG-MEM α . (E, E') A colony with Oct4-GFP expression at 3 days culture after first passage in LG-MEM α . (F, F') A growing colony with Oct4-GFP expression at 3 days after the second passage in LG-MEM α . (G, G') ES cell colonies with Oct4-GFP expression in LG-MEM α (scale bar = 50 μ m).

TABLE 2. PERSISTENCY OF *Oct4*-GFP EXPRESSING CELLS DURING ES CELL DERIVATION AND ES CELL DERIVATION EFFICIENCY FROM PREBLASTOCYSTS BY CULTURE CONDITION, GLUCOSE CONCENTRATION AND EPIGENETIC MODIFICATION

	No. of PB	At 2-h after first passage ^a	At 3 days after first passage ^a	At 3 days after second passage ^a	ES lines (%)
LG-MEM α	20	18	8	5	5 (25.0)
HG-MEM α	20	20	6	5	4 (20.0)
KDMEM	18	17	4	2	1 (5.5)

PB, Preblastocyst; LG-MEM α , MEM α containing low glucose; HG-MEM α , modified MEM α containing high glucose; KDMEM, knockout-DMEM; %, ES cell lines/Preblastocyst.

^aNumber of wells containing *Oct4*-GFP expressing cells.

dissociation and first passage of preblastocysts, we observed some wells containing one to two small cell masses expressing *Oct4*-GFP (Fig. 1D and D') and other wells where cell masses did not express *Oct4*-GFP. Over 90% of wells contained *Oct4*-GFP-expressing cells at 2 h postdissociation, but the percentage of wells containing *Oct4*-GFP expressing cells at later time points differed, depending on the derivation culture conditions. The *Oct4*-GFP-expressing masses were able to proliferate, with GFP expression becoming stronger (Fig. 1E and E') over 4 days of culture. In LG-MEM α , 40% of wells had *Oct4*-GFP-expressing cells at 3 days after first passage, whereas 30% of HG-MEM α and 22.2% of KDMEM wells expressed *Oct4*-GFP (Table 2). After the second passage, many wells contained one to two prominent colonies with ES cell colony-like morphology (Fig. 1F and F'). At 3 days after the second passage, the *Oct4*-GFP expression rates were 25, 25, and 11.1% for LG-MEM α , HG-MEM α , and KDMEM, respectively (Table 2). Wells containing prominent ES cell-like colonies were passaged a third time to multiwell plates (Fig. 1G and G') for expansion. The most efficient derivation of ES cell lines from preblastocysts occurred in the low-glucose environment, LG-MEM α , compared with the high-glucose environments, HG-MEM α , and KDMEM (Table 2) (25, 20, and 5.5%, respectively).

Characterization of ES cells from preblastocyst

ES cells derived from preblastocysts were characterized by immunostaining using anti-SSEA-1 and alkaline phosphatase (AP) antibodies (Fig. 2). All successfully derived ES cell lines from preblastocysts in LG-MEM α , HG-MEM α , and KDMEM expressed SSEA-1 and AP activity (Supplementary Table S1; see online supplementary data at www.liebertonline.com/cell). SSEA-1 was expressed in colonies and colocalized with *Oct4*-GFP expression (Fig. 2A–D). AP was expressed and colocalized with colonies (Fig. 2E–I). Genomic integrity of the ES cell lines was evaluated by karyotyping (Supplementary Table S1). At passage 8, karyotype analysis showed that all lines from preblastocysts had over 70% euploid chromosome count. This percentage is in normal range of reported mouse ES cell lines (Rebuzzini et al., 2008a, 2008b). Teratoma study is very important assay to demonstrate pluripotent cell lines' differentiation and pluripotency. ES cells derived from preblastocysts and cultured in different culture conditions were evaluated for their pluripotency by their potential for differentiation into the three primary germ layers *in vivo*, assayed by teratoma formation after subcutaneous injection of the ES cells into SCID mice (Fig. 3 and Table 3). Histology of teratomas derived from preblastocysts cultured in different culture conditions

FIG. 2. Characterization of preblastocyst ES cells from LG-MEM α by antistage-specific mouse embryonic antigen-1 (SSEA-1) and alkaline phosphatase (AP) activity. ES cells derived from preblastocyst showing (A) anti-SSEA-1 immunostaining; (B, G) *Oct4*-GFP expression; (C, H) DAPI staining. (D) Merged image for A–C. (E) AP staining (bright field); (F) AP staining (fluorescent); (I) Merged images for F–I (scale bar = 50 μ m).

FIG. 3. Histology of teratomas formed by preblastocyst-derived ES cells. (A, D, G) Ectodermal, neuronal epithelium-like tissues; (B, E, H) Endodermal, gut-like tissues; (C, F) Mesodermal, cartilage-like tissues; (I) Mesodermal, muscle-like tissue; LG-MEM α , MEM α containing low glucose; HG-MEM α , modified MEM α containing high glucose; KDMEM, knockout-DMEM (scale bar = 200 μ m).

such as KDMEM (Fig. 3A–C), HG-MEM α (Fig. 3D–F), and LG-MEM α (Fig. 3G–I) was analyzed, and in each case, cells had differentiated into primary three germ layers in teratomas *in vivo*, such as primitive ectoderm (neuronal epithelium-like tissues; Fig. 3A, D, and G), endoderm (gut-like tissues; Fig. 3B, E, and H), and mesoderm (cartilage-like tissues; Fig. 3C and I, and muscle-like tissue; Fig. 3F).

Global gene expression profiles of ES cell lines from preblastocysts and blastocysts are highly similar

One-way ANOVA analysis identified only 22 genes that were differentially expressed across the six ES cell lines derived from preblastocysts or blastocysts in LG-MEM α , HG-MEM α , or KDMEM (Fig. 4 and Supplementary Table S3). Hierarchical clustering on relative expression levels of these 22 genes produced a dendrogram that clearly groups the ES cell lines based on the medium formulation from which they were derived, as opposed to their source embryo developmental stage (Fig. 4). Overall, the six different classes of mouse ES cell lines analyzed had extremely similar gene expression profiles, which were significantly different from that of the MEF control, as expected.

Discussion

The *zona pellucida* is an extracellular matrix surrounding mammalian eggs that blocks polyspermy and protects the developing preimplantation embryo (Boja et al., 2003). It has been reported that early implantation stage embryos from which the *zona pellucida* was removed developed into blastocysts by *in vitro* culture without feeders (Mintz, 1962). In the present study, *zona*-free embryos that were cultured on feeders differed developmentally from intact embryos. To the best of our knowledge, this is the first report of the induction of whole preblastocyst outgrowth from *zona*-free embryos.

Recently, we reported that glucose level affects ES cell derivation efficiency using blastocyst from the same mouse strain of our current study (50% B6, 25% CBA, and 25% DBA), and showed that high glucose level had better support for Oct4-GFP expression and ES cell derivation efficiency (Kim et al., 2009). It was reported that high glucose levels are required for growth of ES cells from 129s train mouse blastocysts *in vitro* (Heilig et al., 2003) and can cause upregulation of mouse ES cell proliferation by activation of cell cycle regulatory proteins (Kim et al., 2006). Otherwise, Wang et al. (2006) reported that low glucose culture conditions increase

TABLE 3. TERATOMA FORMATION FROM PREBLASTOCYST-DERIVED ES CELL LINES

Culture condition	No. of injected mouse	Site of injection	Teratoma (No. of teratoma-formed mice)	Ectoderm	Endoderm	Mesoderm
KDMEM	3	Thigh (1) Back (2)	Yes (3)	Yes	Yes	Yes
HG-MEM α	3	Thigh (1) Back (2)	Yes (3)	Yes	Yes	Yes
LG-MEM α	3	Thigh (1) Back (2)	Yes (3)	Yes	Yes	Yes

FIG. 4. Differential expressing (DE) gene sets across ES cell classes. Hierarchical clustering on a set of 22 probes identified as DE across the six ES cell classes. Vertical branches are color coded based on culture medium used in derivation. See Supplementary Table S3 for gene listing. PB, preblastocyst-derived; BL, blastocyst-derived; KD, knockout DMEM; LG, low glucose-MEM α ; HG, high glucose-MEM α .

the efficiency of ES cell derivation from mouse blastocysts, based on comparisons of basal DMEM containing low (1.0 g/L) or high (4.5 g/L) glucose, and KDMEM. To ask whether glucose levels affect ES cell derivation efficiency from preblastocysts, we used a MEM α (LG-MEM α), and modified it by adding glucose (HG-MEM α) and compared with standard medium containing high glucose (KDMEM). MEM α -based media were able to support more efficient ES derivation, as well as *Oct4*-GFP expression rates in outgrowths after the second passage, compared with KDMEM. Comparing the LG-MEM α and HG-MEM α groups in which the only difference was the glucose concentration, interestingly, there was no significant difference in the efficiency of ES cell derivation (25 and 20%, respectively) or support for *Oct4*-GFP expressing cells (25 and 25%, respectively). LG-MEM α had the best ES cell derivation efficiency of all evaluated conditions in this study from preblastocysts using a hybrid mouse strain (50% B6, 25% CBA, and 25% DBA), and this result is in contrast with our previously report for the efficiency of ES cell line derivation from blastocysts, where HG-MEM α was the most efficient medium for ES cell derivation (Kim et al., 2009). Unexpectedly, we found that KDMEM proved less efficient in terms of ES cell derivation and support of *Oct4*-GFP expressing cells after the second passage. KDMEM, which is commonly used for ES cell derivation and maintenance of mouse and human ES cell lines (Amit et al., 2000; Roach and Mcneish, 2002), contains high glucose (4500 mg/L). It has been reported that early preimplantation stage embryos

(one-cell to morula stage embryos) differ from blastocysts in terms of their glucose consumption because glucose consumption increases dramatically after the morula stage, as the blastocoel forms within the blastocyst (Pinto et al., 2002). The lack of a significant effect of glucose levels for the efficiency of ES cell derivation in the present study may be due to the transition timing of development for glucose uptake. This difference is reflective of the differential energy source usage of mammalian embryos as they shift from pyruvate to glucose as an energy source during cavitation (Gardner et al., 2000).

In the present study, we demonstrated that ES cell lines could be derived from preblastocysts using a hybrid mouse strain (50% B6, 25% CBA, and 25% DBA), which was not previously evaluated for ES cell line derivation from preblastocyst. One of the novelties of this study is the use of a refractory strain of mouse for ES cell derivation from preblastocysts. Our data provide a more efficient protocol enabling derivation of large numbers of ES cell lines from preblastocysts. Based on the global gene expression profiling presented here, it appears that the ES cell lines produced from blastocysts and preblastocysts, regardless of the derivation culture conditions, are essentially indistinguishable by differences in mRNA levels. Most notably, they are indistinguishable from ES cells derived from blastocyst in standard medium, represented here by the BL-KDMEM class with no statistically significant differentially expressed genes identified in pairwise comparisons with the other five ES cell classes (Supplementary Table S2). This high degree of similarity suggests that the gene regulatory networks within these cell lines are programmed in the same way, and is consistent with the idea that the cell lines are functionally equivalent.

The fact that very substantial differences are seen in comparisons between the ES cell lines and MEFs, in the form of large groups of statistically significant DE genes (Table S3), is evidence that the microarray system and the experimental design used here are readily able to detect differences in gene expression profiles where they exist.

Recently, new cell lines, epiblasts stem cells (EpiSCs), were derived from different time point 5.5~5.75 day implanted mouse embryos with high efficiency under conditions used for human ES cell culture, but these cell lines did not exhibit the same degree of pluripotency as standard mouse ES cells and were not able to be derived in mouse ES cell culture medium (Brons et al., 2007; Tesar et al., 2007). Interestingly, EpiSCs grow in a monolayer, like human ES cells, and had much greater overlap of *Oct4*-target genes with human ES cells than mouse ES cells and, surprisingly, had different gene expression levels for germ cell specific genes and stem cell specific genes, compared with mouse ES cells. Comparing the pluripotency and gene expression of human ES cells derived from human preblastocysts, human ES cells derived from human blastocyst, and mouse ES cells derived from preblastocysts may provide information critical to the further development of stem cell research. Researchers have generally focused on ES cell derivation methods developed from the blastocyst stage and applying them to induced pluripotent stem cell (iPS cell) generation. The lack of diversity on developmental stages has restricted the evaluation of pluripotency of derived ES cells by technical difficulties and potential applications. Here, we introduce new methods of ES cell derivation from the preblastocyst stage that may

provide alternative ways to generate iPS cell and understand developmental.

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Data Deposition Footnote

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE17560.

Author Contribution Summary

Chul Kim: conception and research design, collection, and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Joonghoon Park: collection and/or assembly of data, data analysis and interpretation. Tomokazu Amano: collection and/or assembly of data. Ren-He Xu: data analysis and interpretation. Mark G. Carter: conception and research design, collection, and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Xiuchun Tian: conception and research design, final approval of manuscript.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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