Chemical-defined medium supporting the expansion of human mesenchymal stem cells

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Abstract

Objectives: Mesenchymal stem cells (MSCs) have been intensively investigated as to their therapeutic potentials. However, the full chemical-defined medium supporting the isolation and expansion of human MSCs has not been developed yet.

Materials and methods: Here, we developed the full chemical-defined medium, NBVbe medium, via RNA sequencing, bioinformatic analysis, and growth factor screening.

Results: The NBVbe medium contains N2B27 medium with the BSA (bovine serum albumin) replaced by the recombinant human albumin, bFGF (basic fibroblast growth factor), vitamin C, and EGF (epidermal growth factor). The NBVbe medium could support the isolation and expansion of human MSCs from the umbilical cords.

Conclusions: The full chemical-defined medium supporting the isolation and expansion of human MSCs has been developed. This would be helpful for further optimization of the MSC medium, their clinical applications, and molecular characterization.

Keywords: Mesenchymal stem cells, MSCs, Chemical-defined medium, NBVbe medium

Introduction

Mesenchymal stem cells (MSCs) are widely investigated because of their therapeutic potentials. In addition to their abilities to differentiate into multiple cell types, they could orchestrate the immune responses and modulate the tissue microenvironment, resulting in tissue regeneration [1–6]. Their supportive roles in regulating hematopoietic stem cell (HSC) homeostasis also highlight their therapeutic applications in HSC transplantations [7, 8].

Despite the intensive studies on their therapeutic applications, many basic questions remain unsolved, such as the cell identity and origin. Differing from other types of adult stem cells, MSCs could be isolated from many tissues, such as the bone marrow, adipose, and umbilical cord [1, 7]. Whether they have the same origin and function remains unclear. Furthermore, their specific cell surface markers have not been developed so far [9]. Indeed, their definition, function, and therapeutic effects need to be delineated precisely [10].

One of the major obstacles to solving these basic questions is that the full chemical-defined medium supporting the expansion of human MSCs is missing. It has been demonstrated that the human serum and platelet lysate (PL) could support the human MSCs’ expansion in vitro [11, 12]. And the human platelet lysate has been used as the standard culture medium for MSCs’ clinical applications [13, 14]. However, the
batch variation of human PL preparation affects the phenotype and function of MSCs [15, 16]. Furthermore, the human platelet has limited resources. Thus, developing a full chemical-defined medium for human MSCs is critical for both basic biology study and also the clinical applications.

To overcome the inconsistent performance associated with the human platelet lysate, lots of efforts have been made to uncover the critical components of the platelet lysate which supports the MSCs’ expansion [17–24]. Unfortunately, it remains largely unclear what constituents mainly contribute to the MSCs’ proliferation. Here, we would provide an alternative strategy to uncover those critical factors for supporting MSCs’ expansion. And finally, we established the full chemical-defined medium supporting the isolation and expansion of the human MSCs.

Materials and methods

Isolation and expansion of human MSCs with serum-based medium

This study was approved by the ethics committee of Shenzhen University and followed the tenants of the Declaration of Helsinki. The human umbilical cords were collected in 10 min after the baby’s birth and the informed consents were obtained. The umbilical cords were stored in DMEM/High Glucose (Gibco, USA) with antibiotics (500 units/mL of penicillin and 500 μg/mL of streptomycin) on ice and delivered to the lab. They were minced into 1–3 mm³ fragments immediately and incubated in 1 mg/mL collagenase B (STEMCELL Technologies, diluted in DMEM/High Glucose) for 12 h at 37 °C. Then the isolated single cells of human MSCs were expanded in DMEM/High Glucose (Gibco, USA) plus 10% human serum (puriﬁed from the umbilical cord blood), 10 ng/mL bFGF (basic fibroblast growth factor, Peprotech), 50 μg/mL vitamin C (Sigma), and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). The human MSCs were passaged with TrypLE (Thermo Scientiﬁc).

Sample treatment and RNA sequencing

Human MSCs at passage 2 were plated onto p6 plates at the concentration of 5 × 10⁴ cells per well. Then they were incubated with DMEM/High Glucose (Thermo Scientiﬁc), 1% platelet lysate (Merk) in DMEM/High Glucose (Thermo Scientiﬁc) plus 2 U/mL heparin (Sigma), 2% platelet lysate in DMEM/High Glucose plus 2 U/mL heparin, or 5% platelet lysate in DMEM/High Glucose plus 2 U/mL heparin for 5 days. The RNA was extracted with Eastep® Super Total RNA Extraction Kit (Promega). The quality control, library construction, RNA sequencing, and bioinformatic analysis were performed in BGI (Beijing Genomics Institute). The PCA (principal component analysis) was performed by princomp and ggplot2 packages in R. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed by phyper in R. The protein-protein interaction analysis was conducted by DIAMOND and STRING. The expression pattern analysis was performed by Mfuzz package in R. All the analyses were conducted with the online bioinformatic platform Dr. Tom (biosys.bgi.com/) provided by BGI.

Medium components screening

For basal medium screening, the human MSCs at passage 2 were plated onto p12 plates at the concentration of 2 × 10⁴ cells per well. Then they were incubated with indicated mediums for 5 days and the cell counted with hemocytometer. The medium was refreshed every 2 days. The DMEM medium was DMEM/High Glucose (Thermo Scientiﬁc). The FBS medium was composed of DMEM/High Glucose plus 10% FBS (fetal bovine serum, Thermo Scientiﬁc). The N2B27 medium was composed of DMEM/High Glucose plus N2 (Thermo Scientiﬁc) and B27 (Thermo Scientiﬁc). The N2B27-VA medium was composed of DMEM/High Glucose plus N2 (Thermo Scientiﬁc) and B27 without vitamin A (Thermo Scientiﬁc). The NB medium was composed of DMEM/High Glucose plus NB mixture (Table 1). The 5% PL medium was composed of DMEM/High Glucose plus 5% platelet lysate (Merk) and 2 U/mL heparin (Sigma).

| Table 1 Composition of the NB medium (× 50) |
|------------------------------------------|
| **Biotin** | 125 μg/mL |
| DL-alpha-tocopherol acetate | 50 μg/mL |
| DL-alpha-tocopherol | 50 μg/mL |
| Human recombinant albumin | 2 mg/mL |
| Catalase | 125 μg/mL |
| Human recombinant insulin | 410 μg/mL |
| Human transferrin (Holo) | 5 mg/mL |
| Superoxide dismutase | 375 U/mL |
| Corticosterone | 1 μg/mL |
| D-Galactose | 750 μg/mL |
| Ethanolamine | 0.05 μM/mL |
| Glutathione (reduced) | 50 μg/mL |
| L-Carnitine | 100 μg/mL |
| Linoleic acid | 50 μg/mL |
| Linolenic acid | 50 μg/mL |
| Progesterone | 640 ng/mL |
| Putrescine | 1.61 mg/mL |
| Sodium selenite | 900 ng/mL |
| T3 (triiodo-l-thyronine) | 100 ng/mL |
For growth factor or chemical screening, the human MSCs at passage 2 were plated onto p12 plates at the concentration of $2 \times 10^4$ cells per well. The growth factors or chemicals were added into the NB medium and incubated the cells for 5 days. The medium was refreshed every 2 days. The concentration was used as 2 $\mu$M for BIO (6-bromoindirubin–3'-oxime), 50 $\mu$g/mL for VC (vitamin C), 10 $\mu$g/mL for OSM (oncostatin M), PDGF (platelet-derived growth factor)-AA, PDGF-AB, PDGF-BB, bFGF, HGF (hepatocyte growth factor), EGF (epidermal growth factor), IGF (insulin-like growth factor), BDNF (brain-derived neurotrophic factor), NGF (nerve growth factor), VEGFA (vascular endothelial growth factor A), TGFβ2 (transforming growth factor beta 2), IL (interleukin)-1β, IL-6, IL-7, CSF2 (colony-stimulating factor 2), CXCL1 (C-X-C motif chemokine ligand 1), CXCL2, CXCL5, CXCL6, CXCL8, CXCL10, CXCL12, CCL2 (C-C motif chemokine ligand 2), CCL7, CCL11, and CCL20 (all from Peprotech).

**Cell doubling time assessment**

The human MSCs at passage 2 were plated onto p6 plates at the concentration of $5 \times 10^5$ cells per well. Then they were incubated with FBS medium (DMEM/High Glucose plus 10% FBS), 5% PL medium (DMEM/High Glucose plus 5% platelet lysate and 2 U/mL heparin), or NBVbe medium (DMEM/High Glucose plus NB, 50 $\mu$g/mL VC, 10 $\mu$g/mL bFGF and 10 $\mu$g/mL EGF), as separate parallel experiments. Cells were passaged with TrypLE when they reached 80–90% confluence and replated with $5 \times 10^4$ cells per well in p6 plate for continuous expansion.

**Flow cytometry**

The human MSCs, expanded with the NBVbe medium, were dissociated with TrypLE and re-suspended in PBS containing 5% BSA (bovine serum albumin, Sigma). They were incubated with the mouse anti-human antibody CD90-FITC, CD73-FITC, CD105-FITC, HLADR-FITC, CD45-FITC, CD34-FITC, CD19-FITC, CD11b-FITC, or IgG-FITC (all from BD Biosciences) for 30 min. Cells were analyzed with the BD ACCURI C6 PLUS machine after being washed with PBS two times. The data were further analyzed with the FlowJo software.

**Colony formation assay**

The human MSCs were dissociated with TrypLE and diluted with the 5% PL or NBVbe medium at the concentration of $1 \times 10^5$ cell/mL. Then the hanging drops were performed on non-adhesive petri-dishes with 20 $\mu$L per drop. Six hours later, the 5% PL or NBVbe medium was added to the dish and cultured for 7 days. Then the colonies were plated onto 0.1% gelatin-coated dishes for 1 day and stained with crystal violet solution (0.05% crystal violet, 1% formaldehyde, and 1% methanol in PBS) for 20 min.

**Human MSC differentiation and characterization**

The human MSC differentiation and characterization were conducted with StemPro® Adipogenesis Differentiation Kit (Gibco), StemPro® Osteogenesis Differentiation Kit (Gibco), StemPro® Chondrogenesis Differentiation Kit (Gibco), and Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems), according to the instructions.

**Isolation and expansion of human MSCs with the NBVbe medium**

The human umbilical cords were collected in 10 min after the baby’s birth and the informed consents were obtained. The umbilical cords were stored in DMEM/High Glucose (Gibco, USA) with antibiotics (500 units/mL of penicillin and 500 $\mu$g/mL of streptomycin) on ice and delivered to the lab. They were minced into 1–3 mm$^3$ fragments immediately and incubated with indicated dissociation reagents according to the instructions. The dissociation reagents include TrypLE (Gibco), 0.05% Trypsin-EDTA (Gibco), Accutase (STEMCELL Technologies), GCD (Gentle Cell Dissociation Reagent, STEMCELL Technologies), SR (ReLeSR™, STEMCELL Technologies), CDB (Cell Dissociation Buffer, enzyme-free, Gibco), ACF (Animal Component-Free Cell Dissociation Kit, STEMCELL Technologies), Collagenase B (STEMCELL Technologies, diluted in DMEM/High Glucose), and DNase I (Sigma), Dispase II (Sigma). Then the isolated single cells of human MSCs were expanded in the NBVbe medium (DMEM/High Glucose plus NB, 50 $\mu$g/mL VC, 10 $\mu$g/mL bFGF, and 10 $\mu$g/mL EGF). The human MSCs were expanded with TrypLE (Thermo Scientific).

**Statistical analysis**

Data were analyzed using SPSS software for Windows (SPSS Inc.) and are shown as mean ± SEM (standard error of the mean). Student’s t test was used for two-group comparison and 1-way ANOVA for multiple-group comparison with normal data distribution, parametric test, and Tukey’s post hoc tests. A level of $P < 0.05$ was considered statistically significant.

**Results and discussion**

Lots of efforts have been made to uncover the critical components of the platelet lysate for supporting the MSCs’ expansion [17–24]. Unfortunately, it remains largely unclear what constituents mainly contribute to the MSCs’ proliferation. To develop the full chemical-defined medium for supporting the proliferation of human MSCs, we investigated the critical regulators,
which are responsible for the MSCs’ proliferation, through analyzing the responses of the MSCs to the platelet lysate via RNA sequencing and bioinformatic analysis, instead of analyzing the platelet lysate itself. Therefore, the transcriptome of the MSCs was analyzed after exposing to different concentrations of human platelet lysate.

The human platelet lysate had the dose effects on the proliferation of human MSCs (Fig. 1a). The PCA showed that the human MSCs treated with 1% and 2% platelet lysate had similar gene expression patterns while the 5% platelet lysate conferred human MSCs as a very different pattern (Fig. 1b). Therefore, the signal pathways or important genes responsible for human MSC proliferation should also have the dose effects. Gene expression pattern clustering among the samples treated without human platelet (negative control (NC)) or with different concentrations of human platelet lysate (1%, 2%, and 5% PL) was performed (Supplementary Fig. 1). Both upregulation and down-regulation patterns were further analyzed, because the key components of the platelet lysate might have the positive regulations or negative feedback regulations on the gene expression of MSCs. Thus, the KEGG pathway enrichment analysis was performed for genes from clusters 2, 3, 5, 7, 8, 9, and 11 (Fig. 1c, Supplementary Fig. 2). Among all these important pathways activated or suppressed by the human PL, the MAPK (mitogen-activated protein kinase) signal pathway was further studied as it has been demonstrated as one key pathway for cell proliferation [25]. Protein-protein interaction network analysis showed that a panel of important growth factors or receptors was involved in the MAPK pathways which might contribute to the supportive role of PL for human MSC proliferation (Fig. 1d).

![Figure 1](image-url)
To analyze these potential important growth factors uncovered from the RNA-sequencing analysis, several basic mediums were firstly tested. Ideally, the basic medium should support the human MSCs alive or proliferation and also have the potential to develop as a full chemical-defined medium. The N2B27 medium is a serum-free medium suitable for neural stem cell and pluripotent stem cell proliferation [26, 27]. Thus, two types of N2B27 were compared, with or without vitamin A. Data showed that the N2B27-VA (N2B27 without vitamin A) could support the human MSC proliferation, much better than the N2B27 with vitamin A or FBS (fetal bovine serum) (Fig. 2a). Although the recipe of commercial N2B27 is unavailable to the public, the more original recipe and modified version of N2B27 had been published [28, 29]. Then the BSA (bovine serum albumin) component of the N2B27-VA was replaced by recombinant human albumin, termed as the NB medium (Table 1). Data showed that the NB medium was comparable to the N2B27-VA medium (Fig. 2A). Then 12 growth factors uncovered from the RNA-sequencing analysis were added to the NB medium to assess whether they could support the human MSC proliferation. Furthermore, we also tested two factors, BIO (6-bromoindirubin-3′-oxime, the GSK3β inhibitor) and OSM (oncostatin M) that were used for cardiac progenitor derivation which also expresses CD105 [30]. Totally, 14 growth factors or chemicals were evaluated. Among them, the Vc (vitamin C), PDGF-AB (platelet-derived growth factor AB), bFGF (basic fibroblast growth factor), and EGF (epidermal growth factor) showed beneficial effects on MSC proliferation while BIO, OSM, and PDGF-

![Fig. 2](https://example.com/fig2.png)
BB significantly suppressed their expansion (Fig. 2b). The four-factor combination (4F), including Vc, PDGF-AB, bFGF, and EGF, showed similar beneficial effects on MSC proliferation when compared with combining them together (the eleven-factor combination, excluding BIO, OSM, and PDGF-BB, 11F) (Fig. 2c). And further eliminating the PDGF-AB did not affect the MSCs proliferation while eliminating the Vc, bFGF or EGF significantly suppressed their proliferation (Fig. 2c). Thus, the 3F (3 basic factors), including Vc, bFGF, and EGF, is the minimal combination for supporting the human MSCs’ expansion, as eliminating anyone of them could

Fig. 3 Characterization of human MSCs cultured with the NBVbe medium. a The human MSCs cultured with NBVbe medium had the different cell morphology from them cultured with the 5% PL medium. Scale bar, 100 μm. b Cell surface marker analysis by flow cytometry. c Colony formation assay for human MSCs cultured with the NBVbe medium or 5% PL medium. Scale bar for the upper panel, 1 cm. Scale bar for the lower panel, 200 μm. d Colony number counting for human MSCs cultured with the NBVbe medium or 5% PL medium for 5 passages. e The human MSCs were directed to adipocytes, osteocytes, and chondrocytes after cultured with the NBVbe medium for 5 passages. Left: Oil Red O staining and anti-FABP4 immuno-staining for the differentiated adipocytes; middle: Alizarin Red S staining and anti-Osteocalcin immunostaining for the differentiated osteocytes; right: Alcian blue staining and anti-Aggrecan immunostaining for the differentiated chondrocytes. Scale bar, 200 μm. An asterisk indicates *P < 0.05
significantly inhibit the cell proliferation (Fig. 2d). Although the NB medium plus 3F, termed as the NBVbe medium, had longer doubling time than the 5% PL, it was much better than the FBS-based medium (Fig. 2e).

To further confirm that the NBVbe medium could really support the expansion of human MSCs, human MSCs were characterized after cultured with the NBVbe medium for 5 passages. They showed more fibroblast-like morphology (Fig. 3a) and were positive for MSC cell markers (Fig. 3b).

**Fig. 4** The RNA profile comparison between the human MSCs cultured with NBVbe or 5% PL. a Venn diagram showing the numbers of genes differentially regulated in human MSCs cultured with NBVbe, 1% PL, 2%PL, or 5% PL medium. b Hierarchical clustering analysis of mRNA profiles in human MSCs cultured with NBVbe, 1% PL, 2%PL, or 5% PL medium. c Differentially expressed genes between the human MSCs cultured with NBVbe and 5% PL. d KEGG pathway enrichment for differentially expressed genes between the human MSCs cultured with NBVbe and 5% PL. e Alternative splicing events detected by RNA sequencing. f Alternative splicing events compared with the human MSCs cultured with DMEM via RNA sequencing analysis. g SNV (single-nucleotide variant) and InDel (insertion and deletion mutations) detected by DNA exon sequencing. NC, negative control, human MSCs cultured with DMEM; DEGs, differentially expressed genes; A5SS, alternative 5′ splicing site; A3SS, alternative 3′ splicing site; MXE, mutually exclusive exons; RI, retained intron; SE, skipped exon.
Fig. 5 (See legend on next page.)
surface marker CD73, CD90, and CD105 while negative for HLA-DAR, CD45, CD34, CD19, and CD11b (Fig. 3b). The human MSCs cultured with the NBVbe medium could form more colonies than the platelet lysate medium (Fig. 3c, d) and differentiate into adipocytes, osteocytes, and chondrocytes (Fig. 3e).

Because the MSCs cultured in the NBVbe medium were less proliferative than the PL medium, we were wondering whether further improvements could be made. RNA sequencing showed that the MSCs cultured with the NBVbe medium had a very different gene expression pattern when comparing them in culture with the NBVbe medium (Fig. 4a). Indeed, there were still a large amount of differentially expressed genes between the MSCs cultured in the NBVbe medium and 5% PL (Fig. 4c). KEGG pathway enrichment analysis for these differentially expressed genes indicated that they were mostly involved into the cell cycle and DNA replication pathways (Fig. 4d). And gene clustering analysis indicated that the NBVbe medium might be more closed to the medium containing 1% platelet lysate (Fig. 4b).

Interestingly, the DNA repair pathway was also activated by the PL medium (Fig. 4d), which indicated that the 5% PL might induce genome instability, the adverse effects alongside the fast cell proliferation. RNA sequencing also revealed that the cells cultured with 5% PL had more alternative splicing events than those cells cultured with the NBVbe medium (Fig. 4e, f). Furthermore, the DNA exon sequencing revealed that the MSCs cultured with 5% PL might have more SNP (single-nucleotide polymorphism) events (Fig. 4g). Thus, the NBVbe medium might introduce less DNA mutations than PL.

In conclusion, we have developed the full chemical-defined medium for isolation and expansion of the human MSCs. The human MSCs cultured with the NBVbe medium had the self-renewal and differentiation abilities with expressing MSC markers, although the bFGF and vitamin C had been demonstrated as effective factors to support the human MSCs differentiation in the conventional medium [31–33]. The NBVbe medium we developed here is the first chemical-defined medium supporting the proliferation of human MSCs with the formulation available to the public. Moreover, this would be very helpful for molecular mechanism studies of the human MSCs, including the cell identity characterization. However, the NBVbe medium was not as good as the human platelet in supporting the proliferation of human MSCs. Thus, more effects should be made to further optimize the chemical-defined medium.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13287-020-01641-7.

Additional file 1: Figure S1. The top 12 expression pattern clusters analyzed by Mfuzz package in R (EPS 14642 kb).
The authors declare that they have no competing interests.

Consent for publication

This study was approved by the ethics committee of Shenzhen University.

Availability of data and materials

All related data are available under request.

Ethics approval and consent to participate

This study approved by the ethics committee of Shenzhen University and followed the tenants of the Declaration of Helsinki. Informed consent to participate in the study was obtained from the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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