Bone marrow-derived humoral factors suppress oxidative phosphorylation, upregulate TSG-6, and improve therapeutic effects on liver injury of mesenchymal stem cells

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Mesenchymal stem cells, which have the potential to be used in regenerative medicine, require improvements in quality for patient use. To maintain stemness of cultured bone marrow-derived mesenchymal stem cells, we focused on the bone marrow microenvironment, generated a conditioned medium of whole bone marrow cells (BMC-CM), and assessed its effects on bone marrow-derived mesenchymal stem cells. BMC-CM suppressed morphological deterioration and proliferative decline in cultured bone marrow-derived mesenchymal stem cells. BMC-CM suppressed oxidative phosphorylation activity, a stemness indicator, and upregulated suppressors of oxidative phosphorylation such as hypoxia-inducible factor 1-alpha, Sirtuin 3, 4, and 5. Furthermore, BMC-CM upregulated TNF-stimulated gene 6 mRNAs, which are considered OXPHOS suppressors. Furthermore, BMC-CM upregulated TNF-stimulated gene 6 protein (TSG-6) production, a central factor underlying the therapeutic effects of MSC, and analyzed the therapeutic effects of BM-MSCs on carbon tetrachloride (CCL₄)-induced liver injury in rats. Furthermore, since numerous recent studies have highlighted the changes in disease pathophysiology based on microRNAs (miRNAs), we investigated exosomal miRNAs derived from whole BM cells for quality improvement of MSC.

**Key Words:** mesenchymal stem cell, oxidative phosphorylation, TNF-stimulated gene 6, microRNA, liver disease

Mesenchymal stem cells (MSCs) are a promising source of cells for regenerative medicine of various organs including the liver, and clinical trials have been widely performed to explore their potential.⁴¹ Furthermore, given the increasing number of reports on the therapeutic potential of MSC,⁴⁻⁵ MSC therapies are expected to be developed for more diseases, including those with acute-stage conditions or excessive immune responses.⁵⁻⁷

We have been developing liver regenerative therapies using bone marrow (BM) cells, and we previously reported the clinical effectiveness of autologous bone marrow infusion (ABMI) therapy.⁶⁻⁸ Currently we are developing a less invasive liver regenerative therapy with cultured autologous BM-derived MSCs (BM-MSCs), and also attempting to develop new methods to culture higher-quality MSCs.

One of the most prominent issues in the development of regenerative therapies using MSCs is quality decrease during culturing. Although human BM-MSCs are readily cultured as adherent cells from bone marrow fluid, long-term subculture have been reported to result in deformation, enlargement, and loss of proliferative capacity in cells.⁹⁻¹² Whilst mouse- and rat-derived BM-MSCs can be isolated and harvested using similar protocols used for human,¹³⁻¹⁵ changes in cell deformation and proliferative capacity are more pronounced in mouse and rat.

In this study, we created a conditioned medium with whole BM cells (BM cells-conditioned medium; BMC-CM) to reproduce the BM microenvironment. The qualitative changes in BM-MSCs cultured in BMC-CM were evaluated by analyzing mitochondrial oxidative phosphorylation (OXPHOS) activity, which is considered an index of stemness, and changes in hypoxia-inducible factor 1-alpha (HIF-1α), Sirtuin 3, 4, and 5 (Sirt3, Sirt4, and Sirt5) mRNAs, which are considered OXPHOS suppressors. Furthermore, we evaluated TNF-stimulated gene 6 protein (TSG-6) production, a central factor underlying the therapeutic effects of MSC, and analyzed the therapeutic effects of BM-MSCs on carbon tetrachloride (CCL₄)-induced liver injury in rats. Furthermore, we investigated exosomal miRNAs derived from whole BM cells for quality improvement of MSC.

**Materials and Methods**

**Isolation and culturing of rat BM-MSCs and preparation of BMC-CM.** Six-week-old male Wistar rats were purchased from Japan-SLC (Shizuoka, Japan). Rat BM-MSCs were cultured, as described previously (Fig. 1A).¹⁷ The femur and tibia of 8–10-week-old rats were excised, and the intramedullary cavity was flushed with Dulbecco’s modified Eagle medium (DMEM) (11885-084; Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (172012; Sigma-Aldrich, St. Louis, MO), from now on named “basal medium”. Bone fragments were crushed in a mortar and washed with phosphate-buffered saline (PBS) (14190-144; Gibco) and treated with DMEM supplemented with 2% collagenase (038-22363; Wako, Osaka, Japan) and 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (17514-15; NACALAI TESQUE, Kyoto, Japan) at 37°C for 1 h to detach the adherent cells. The resulting cell suspension was

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Fig. 1. Culturing of BM-MSCs and preparation of BMC-CM. BM-MSCs obtained from the cell suspension of collagenase-treated crushed compact bones were cultured. BMC-CM was prepared by culturing whole bone marrow cells for 3 days. The BM-MSCs obtained as adherent cells were subcultured in basal medium or BMC-CM (A). Almost all the adherent cells at the time of passage 1 in each medium exhibited surface antigens matching MSCs (B). Adherent cells cultured in basal medium at passage 1 were capable of differentiating into adipocytes, osteocytes, and chondrocytes (C). No significant difference was found between cells cultured in basal medium and those cultured in BMC-CM in terms of surface antigen pattern and differentiation ability. Cell growth curve of cells at passage 1 in each medium analyzed by IncuCyte® ZOOM (D) and cell counts by Infinite® TECAN (E) revealed that higher proliferation ability was maintained in long-term subculture in BMC-CM. BMC-CM also maintained the cell morphology and colony forming ability of MSCs during long-term subculture (F, G). Error bars indicate SE. *p<0.05 in t test. **p<0.01 in t test.
filtered using a 70-μm cell strainer (352350; BD Biosciences, Franklin Lakes, NJ) and centrifuged at 330 g for 5 min. The cell pellet was resuspended in basal medium, seeded in a culture dish (353046; Corning, Corning, NY), and cultured at 37°C and 5% CO₂. During the first 3 days, the medium was frequently exchanged every 8 h to eliminate floating cells. At 96 h post-seeding, adherent cells were sub-cultured by adding trypsin (0.25% Trypsin-EDTA, 25200-072; Gibco) in basal medium, BMC-CM or BMC-CM filtered with a 20-nm filter provided in a commercially available exosome-fractionation kit (ExoMIR® PLUS; Bioo Scientific, Austin, TX), from now on named “filtered BMC-CM” (Fig. 4A). Media were exchanged every 2 days and cells were sub-cultured until they were 100% confluent.

BMC-CM was prepared using a cell suspension containing whole BM cells obtained through flushing of the bone medullary space. Cells were resuspended in basal medium at 10⁶ cells per 100 ml, cultured in a T175-flask (353112; Corning) at 37°C and 5% CO₂ for 72 h, and centrifuged at 330 g for 5 min. The supernatant was filtered through a 220-nm filter (MILLEX® GP; Merck, Kenilworth, NJ) and used as BMC-CM (Fig. 1A).

**Assays for cell viability and differentiation.** Cells at passages 1, 3, and 5 in each medium were seeded in 96-well plates (351172; Corning) at 1,000 cells per well, and cell proliferation was evaluated at 37°C and 5% CO₂ using a live-cell imaging system (IncuCyte® ZOOM; Essen Bioscience, Tokyo, Japan), followed by cell counting at 4 days after seeding in a fluorescence plate reader (Infinite M200 PRO; TECAN, Mannedorf, Switzerland) for the cell proliferation assay (CyQUANT® plate reader). Furthermore, cells were seeded in a 100-mm dish (353003; Corning) at 100 cells per dish, cultured at 37°C and 5% CO₂ for 7 days, fixed with 10% formalin (11-0705-7; Sigma-Aldrich), and the fibroblast-like colonies were observed after Giemsa staining (colony-forming unit fibroblast assay; CFU-f assay).

Cells at passage 1 were cultured with commercially available differentiation-inducing reagents (SC020; R&D Systems, Minneapolis, MN), and the differentiation potential to the fibroblast-like colonies was evaluated at 37°C and 5% CO₂, media were exchanged with XF Assay Medium (Seahorse Biosciences) supplemented with 25 mM glucose, and OCR measurements were carried out over 5-min periods, following a 3 min mix period. Cells were treated via sequential addition of 1 µg/ml oligomycin, 300 nM cytochrome c, and 1 µM rotenone. The basal OCR was normalized to the cell number, and the spare respiratory capacity and the maximal respiration were determined in accordance with the manufacturer’s instructions (Seahorse Biosciences).

**Table 1. Primers used in this study**

| Gene name | NCBI reference | Primer sequence | Product size (bp) |
|-----------|----------------|-----------------|-----------------|
| Gapdh     | NM_017008      | Forward: 5’-GGCAAGTTCAACGGCAGCTC-3’ | 96              |
|           |                | Reverse: 5’-AGCACACGATCACCCATT-3’ |                 |
| Hif1a     | NM_0024359.1   | Forward: 5’-TCTAGTGAAAGGATGGAG-3’ | 96              |
|           |                | Reverse: 5’-TCGTTAATGTCAGTGTTGGA-3’ |                 |
| Sirt3     | NM_001106313.2 | Forward: 5’-TGACACGGTCTGCAAGCTG-3’ | 83              |
|           |                | Reverse: 5’-ATGTCAGGTTTCACAGGCCAGTA-3’ |               |
| Sirt4     | NM_001107147.1 | Forward: 5’-CTGCTGCCCTTAAATAGACACCACA-3’ | 117             |
|           |                | Reverse: 5’-GATGCAAGACAGTAAAGCTCCCA-3’ |                 |
| Sirt5     | NM_001004256.1 | Forward: 5’-GCAAGGGTGTGGTCTCATG-3’ | 106             |
|           |                | Reverse: 5’-CAGGAAATCAGGCAACACCGA-3’ |                 |
| Vegfa     | NM_001110333.2 | Forward: 5’-CTCTTGGCTCATACCAGGTACTG-3’ | 103             |
|           |                | Reverse: 5’-ACAGTGGTGGCCCCAGTCCA-3’ |                 |
| Hk2       | NM_012735.2    | Forward: 5’-TGGATGGGATTCGTCAACAGAAGA-3’ | 65              |
|           |                | Reverse: 5’-ACACTACAGTCGGGACCCACAGG-3’ |               |
| Tsg6 (Tnfalpha) | NM_05382.1 | Forward: 5’-CGTCTTGGCAACTCAACAGCAGCTA-3’ | 100             |

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analyzed using mRNA-microarray chips (3D-Gen®; TORAY, Tokyo, Japan). The results of the pathway analysis were integrated via Ingenuity Pathway Analysis (IPA) (QIAGEN).

**Western blot analysis.** Protein lysates were obtained from cell pellets at passage 1 using a commercially available cell lysis buffer (98035; Cell Signaling Technology, Danvers, MA) containing protease inhibitors (complete Mini; Roche Diagnostics GmbH, Mannheim, Germany), mixed with sample buffer containing sodium dodecyl sulfate (SDS) (161-0737; BIO-RAD, Hercules, CA) at a ratio of 1:1 (v/v), followed by boiling for 5 min. Western blot analysis was performed with purified polyclonal anti-rabbit IgG against TSG-6 (PA5-67008; Invitrogen) at 1,000-fold dilution, and monoclonal anti-β-actin antibody produced in mouse (A5316; Sigma-Aldrich) at 5,000-fold dilution as the primary antibodies, and horseradish peroxidase-linked secondary antibodies from donkey against rabbit IgG (NA934; GE Healthcare, Little Chalfont, UK) or from sheep against mouse IgG (NA931; GE Healthcare) at 5,000-fold dilution. Casein-based blocking reagent (1 BlockTM; Invitrogen) dissolved at a concentration of 1% in the basal buffer, PBS with 0.1% tween (160-21211; Wako), was used as the blocking buffer. Imaging and data-analysis were performed using the ChemiDoc™ system (BIO-RAD) and Image Lab™ software (BIO-RAD).

**Animal studies.** All animals were maintained in accordance with the animal care guidelines of Yamaguchi University, and all animal experiments received approval (No. 21-044). After 1 week of acclimatization, twenty-four 8-week-old male Wistar rats, were intraperitoneally administered CCl₄ (037-08075; Wako) diluted 2-fold with corn oil (032-17016; Wako) at 0.5 ml/kg body weight twice a week for 8 weeks. Rats were divided into three groups: control, basal, and BMC-CM groups. The basal and BMC-CM groups were administered 1 ml of 10⁶ BM-MSCs in PBS cultured in basal medium or BMC-CM at passage 1, and the control group was administered 1 ml PBS at 5, 6, and 7 weeks via the tail vein. After 8 weeks, the rats were euthanized via 4% isoflurane in basal medium or BMC-CM at passage 1, and the control group was administered 1 ml of 10⁶ BM-MSCs in PBS cultured in basal medium or BMC-CM at passage 1, and the control group was administered 1 ml PBS at 5, 6, and 7 weeks via the tail vein. After 8 weeks, the rats were euthanized via 4% isoflurane anesthesia, blood was sampled from the inferior vena cava, and the liver was excised (Fig. 3A).

Serum albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured using a biochemical measurement device (SpotChem™ D-02; Arkley. Kyoto, Japan). Liver tissues were fixed in formalin, embedded in paraffin, cut into 3-μm-thick sections, and deparaffinized. Hematoxylin-eosin (H-E) staining was performed with 1% Sirius-red solution (3306-1; MUTO PURE CHEMICALS, Tokyo, Japan) containing Van Gieson Solution P (19,20) and the number of vacuolated hepatocytes and of TUNEL-positive cells and the ratio of Sirius-red stained area were determined.

**Identification of miRNAs corroborating the effects of BMC-CM.** Exosomal miRNAs in BMC-CM cultured for 3, 12, and 72 h were extracted using a commercially available kit with combination filters (ExoMin™PLUS) containing a set of 20-nm and 220-nm filters. These miRNAs were analyzed using a miRNA microarray chip (3D-Gen®; TORAY), and 49 miRNAs were upregulated over time in the BMC-CM samples (Table 2). Seven of these miRNAs targeted numerous genes related to the change of OXPHOS activity observed in the mRNA microarray of BM-MSCs cultured in 20-nm-filtered BMC-CM vs BMC-CM, which were selected in accordance with the "microRNA Target Filter" function of IPA (Table 3). (19,20) Agonists, and the associated negative controls (mirVana™; Life Technologies, Carlsbad, CA) for each of the selected seven miRNAs were purchased and

| Table 2. Increasing miRNA expression in the BMC-CM incubation process |
|---------------------------|-------------------------|-----------------------------|
| miRbase ID               | 12 h vs 3 h (fold)    | 72 h vs 3 h (fold)           |
|---------------------------|-------------------------|-----------------------------|
| rno-miR-666-5p            | 4.26                    | 29.79                       |
| rno-miR-92b-3p            | 2.15                    | 17.94                       |
| rno-miR-92a-3p            | 1.57                    | 12.45                       |
| rno-miR-291a-5p           | 2.26                    | 8.24                        |
| rno-miR-25-5p             | 1.22                    | 6.69                        |
| rno-miR-486              | 1.47                    | 6                           |
| rno-miR-375-5p           | 2.71                    | 5.58                        |
| rno-let-7d-3p            | 1.74                    | 4.61                        |
| rno-miR-23a-3p           | 0.95                    | 3.39                        |
| rno-miR-128-3p           | 0.95                    | 3.01                        |
| rno-miR-221-3p           | 1.07                    | 2.99                        |
| rno-miR-23b-3p           | 0.99                    | 2.98                        |
| rno-miR-294              | 1.56                    | 2.94                        |
| rno-miR-181b-5p          | 1.75                    | 2.9                         |
| rno-miR-25-3p            | 1.06                    | 2.8                         |
| rno-miR-3473            | 1.24                    | 2.74                        |
| rno-miR-290              | 2.15                    | 2.71                        |
| rno-miR-505-3p          | 2.1                     | 2.66                        |
| rno-miR-212-3p           | 1.33                    | 2.4                         |
| rno-miR-874-3p           | 2.11                    | 2.34                        |
| rno-miR-702-5p           | 1.34                    | 2.2                         |
| rno-miR-326-5p           | 1.99                    | 2.2                         |
| rno-miR-145-5p           | 0.9                     | 2.18                        |
| rno-miR-378a-5p          | 1.72                    | 2.11                        |
| rno-miR-10a-5p           | 0.8                     | 2.03                        |
| rno-miR-339-5p           | 1.36                    | 2.02                        |
| rno-miR-22-3p          | 0.86                    | 1.97                        |
| rno-miR-1188-3p         | 1.51                    | 1.93                        |
| rno-miR-328a-3p         | 1.07                    | 1.89                        |
| rno-miR-328b-3p         | 1.07                    | 1.83                        |
| rno-miR-99b-5p           | 0.92                    | 1.8                         |
| rno-miR-532-3p           | 1.68                    | 1.77                        |
| rno-miR-99a-5p           | 0.77                    | 1.77                        |
| rno-miR-3594-3p         | 1.56                    | 1.72                        |
| rno-miR-27a-3p           | 0.83                    | 1.68                        |
| rno-miR-425-5p           | 0.7                     | 1.66                        |
| rno-miR-598-5p           | 3.55                    | 1.56                        |
| rno-miR-484            | 1.38                    | 1.54                        |
| rno-miR-551b-3p         | 0.69                    | 1.53                        |
| rno-miR-100-5p            | 0.71                   | 1.51                        |
| rno-miR-1247-3p          | 2.81                    | 1.46                        |
| rno-miR-3085           | 1.75                    | 1.46                        |
| rno-miR-204-3p           | 1.68                    | 1.37                        |
| rno-miR-3593-3p         | 1.9                     | 1.12                        |
| rno-miR-128-1-5p        | 3.59                    | 1.08                        |
| rno-miR-6318           | 2.21                    | 0.94                        |
| rno-miR-350             | 1.65                    | 0.92                        |
| rno-miR-128-2-5p        | 2.32                   | 0.74                         |
| rno-miR-652-5p           | 1.55                   | 0.68                         |

Under bar: miRNAs selected for the transduction experiment on BM-MSC. †miRNAs that can decrease in liver cirrhosis. ‡miRNAs reported to decrease with fat deposition of HepG2 cells. doi: 10.3164/jcbn.19-125
transfected into BM-MSCs cultured in the basal medium at passage 1 via culturing with transfection reagents (Lipofectamine 2000). The transfection efficiency was confirmed by flow cytometry and real-time PCR. The expression of the transfection marker was analyzed using a fluorescence-activated cell sorter (FACS) and qPCR. The results were analyzed using statistical software (GraphPad Prism) to determine the significance of differences. The data are presented as mean ± standard error of the mean (SEM) values.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM) values. Statistical analysis was performed using the JMP Pro software (SAS Institute, Cary, NC). The results were analyzed using Student’s t-test and one-way analysis of variance (ANOVA) with post hoc analysis using Turkey’s multiple comparison test. Differences were considered significant at p values < 0.05. All results except microarrays for miRNAs and mRNAs presented are from at least three independent experiments for each condition.

Results

BMC-CM improves the viability of BM-MSCs. At the first passage, very few adherent cells were found to express the leucocyte common antigen CD45, hematopoietic progenitor cell marker CD34, or monocyctic lineage marker CD11b. In contrast, nearly all cells expressed MSC markers, including CD54 and CD90 (Fig. 1B). Furthermore, cells differentiated into adipocytes, osteocytes, and chondrocytes (Fig. 1C). Thus, adherent cells at the first passage displayed considerably sufficient purity of MSCs for subsequent experiments. No significant difference was observed between cells cultured in basal medium and those cultured in BMC-CM in terms of their surface antigen pattern and differentiation potential.

Cultured cells initially exhibited small spindle-shaped morphology; however, those cultured in basal medium rapidly transformed into large spherical cells upon repeated sub-culturing (Fig. 1F). Although the growth potential peaked at passage 3 in each medium, a marked reduction in growth potential was subsequently observed in basal medium. In contrast, the morphology and proliferative capacity of the cells were retained for a longer period of time in BMC-CM (Fig. 1D–F). Results from CFU-f assay revealed that the colony forming potential was reduced and lost upon subsequent sub-culturing in basal medium, whereas in BMC-CM, the number and size of colonies were maintained for a longer period (Fig. 1G).

BMC-CM suppresses mitochondrial OXPHOS activity and enhances TSG-6 expression of BM-MSCs. Subsequently, we evaluated the changes in mitochondrial OXPHOS activity as an index of the stemness in cultured BM-MSCs. OCR measurements with an extracellular flux analyzer revealed that the maximal respiration (0.52-fold, p = 0.001) and the spare respiratory capacity (p < 0.001) were significantly suppressed in BMC-CM (Fig. 2A). We further evaluated RT-PCR for mRNA expression levels of various proteins that control the OXPHOS activity. Expression of Hif1α was decreased upon repeated sub-culturing in basal medium but it was increased in BMC-CM. Since HIF-1α protein is rapidly degraded in the presence of oxygen, it was difficult to quantify the protein concentration. However, due to increased mRNA expression of downstream targets of HIF-1, including Vegfa and Hk2, the HIF-1 signaling pathway was suggested to be activated by BMC-CM. We further evaluated the changes in mRNA expression of Sirt3-5 co-localized with the mitochondria, which is considered a modulator that suppresses the mitochondrial OXPHOS activity (21). Expression of Sirt3, Sirt4, and Sirt5 in basal medium peaked at passage 3, while upregulation continued to increase in BMC-CM (Fig. 2B).

Furthermore, we evaluated the variation of Tsg6, which is considered as a quality index of MSC from the viewpoint of the treatment mechanism for liver injury. Although the Tsg6 expression peaked at passage 3 and then decreased regardless of the medium, in passage 1 and 3, the expression was significantly enhanced in BMC-CM-cultured cells (Fig. 2B). Tsg6 protein expression at passage 1 was significantly increased in cells cultured in BMC-CM (4.08-fold, p = 0.013), as demonstrated by Western blot analysis (Fig. 2C).

BMC-CM improves the viability of BM-MSCs. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student’s t-test and one-way analysis of variance (ANOVA) with post hoc analysis using Turkey’s multiple comparison test. Differences were considered significant at p values < 0.05. All results except microarrays for miRNAs and mRNAs presented are from at least three independent experiments for each condition.
them, seven miRNAs, miR-1247-3p, miR-204-3p, miR-128-1-5p, miR-92b-3p, miR-23b-3p, miR-326-5p, and miR-145-5p, were considered to be involved in the change of mitochondrial OXPHOS activity between the cells cultured in 20-nm-filtered BMC-CM and BMC-CM (Table 3), based on the data of mRNA-microarray of cultivated BM-MSCs and information regarding the target genes obtained from the IPA database (using “microRNA Target Filter” function of IPA).

By transfecting the selected seven miRNAs into BM-MSCs, the following trends in upregulation were observed (Fig. 5A): miR-92b-3p (1.82-fold, \( p = 0.002 \)), miR-23b-3p (1.20-fold, \( p = 0.029 \)), miR-204-3p (1.29-fold, \( p = 0.007 \)), and miR-1247-3p (1.33-fold, \( p = 0.038 \)) enhanced the expression of Hif1a; miR-92b-3p (1.64-fold, \( p < 0.001 \)), miR-23b-3p (1.53-fold, \( p = 0.021 \)), and miR-204-3p (1.60-fold, \( p = 0.022 \)) enhanced Sirt3 expression; miR-92b-3p (1.68-fold, \( p = 0.001 \)) and miR-23b-3p (1.18-fold, \( p = 0.048 \)) enhanced Sirt4; and miR-23b-3p (1.34-fold, \( p = 0.0197 \)), miR-204-3p (5.25-fold, \( p < 0.001 \)), miR-1247-3p (4.77-fold, \( p = 0.002 \)), and miR-326-5p (3.92-fold, \( p = 0.003 \)) upregulated the expression of Tsg6.

### Discussion

In this study, we first tried to improve the quality of cultured BM-MSCs through reconstruction of the BM microenvironment maintaining stemness of MSC, and found the OXPHOS-inhibitory
Fig. 3. Administration of BM-MSCs to rats with CCl4-induced liver injury. Male Wistar rats (8-week-old) were intraperitoneally administered 0.5 ml/kg CCl4 twice a week for 8 weeks and treated with BM-MSCs cultured in basal medium or BMC-CM on weeks 5, 6, and 7 (A). In the blood biochemistry examination, the BMC-CM group showed a significant reduction in serum AST and ALT levels compared with that shown by the basal group (B). The ratio of the fibrotic area evaluated with Sirius-red staining, the number of vacuolated hepatocytes, and the number of TUNEL-positive cells were also reduced in the BMC-CM group compared with the basal group (C). Error bars indicate SE. *p<0.05 in t test. **p<0.01 in t test.
Fig. 4. Effects of removing particles over 20 nm from BMC-CM. Using the ExoMir™ PLUS kit, particles over 20 nm were removed from BMC-CM (filtered BMC-CM), and exosomal microRNAs contained in BMC-CM were extracted (A). Mitochondrial OXPHOS activity was accelerated, and the spare respiratory capacity was significantly increased in filtered BMC-CM compared with non-filtered BMC-CM in cells at passage 1 (B). Expression of Hif1α tended to decrease, and that of Sirt3, Sirt4, and Sirt5 were significantly reduced by filtered BMC-CM in cells at passage 3 (C). Pathway analysis of mRNA-microarray data from BM-MSCs cultured in BMC-CM vs filtered BMC-CM analyzed by IPA revealed that 20–220-nm particles were involved in multiple signaling pathways such as OXPHOS, Sirtuin signaling, mTOR signaling, PI3K/Akt signaling, and IGF-1 signaling pathways (D). The overlap p value (p value), indicator of overlap between observed gene expression changes and known targets regulated by transcriptional regulators, was calculated using Fisher’s exact test. The activated z-score (z-score), indicator of regulation direction of pathways, was calculated based on the database of molecular network that represents experimentally observed gene expression or transcription events; positive z-score means “activating” and negative “inhibiting”. OXPHOS-pathway map by pathway analysis of IPA showed that all of the mitochondrial complexes I to V were suppressed by 20–220-nm particles in BMC-CM. Up-regulated components are colored red, and down-regulated green (E). Error bars indicate SE. *p<0.05 in t test. **p<0.01 in t test.
effect of BMC-CM. High-quality stem cells, retaining their stemness, are known to suppress mitochondrial OXPHOS and depend on glycolysis.\cite{22} One of the most important enzymes in such cellular metabolic reprogramming of stem cells is HIF-1α, which is upregulated in a hypoxic environment such as BM, suppresses mitochondrial OXPHOS activity and activates the anaerobic glycolytic metabolic pathway.\cite{23,24} HIF-1α has been further demonstrated to participate in various cell functions such as proliferation, angiogenesis, and immunoregulation, and is currently considered as one of the master regulators of the stemness.\cite{25–27}

Therefore, we have demonstrated the upregulation of HIF-1α upon culturing in BMC-CM under normoxic conditions. Intra-cellular upregulation of HIF-1α under hypoxia is generally considered to be primarily due to the oxygen-dependence of its degrading enzyme, but not from an increase in its production.\cite{28,29} In contrast, multiple pathways including growth factors upregulating HIF-1α regardless of the oxygen concentration, such as IGF-1 or mTOR signaling pathways, have also been reported.\cite{30–34}

Fig. 5. Whole bone marrow cell-derived miRNAs may improve the therapeutic quality of MSCs. Of the seven miRNAs used for transfection experiments, miR-23b-3p, miR-92b-3p, miR-204b-3p, miR-326b-5p, and miR-1247b-3p significantly upregulated one or more mRNA (Hif1a, Sirt3, Sirt4, Sirt5, and Tsg6) (A). There were many common miRNAs involved in OXPHOS suppression and in increased TSG-6 expression. Of these five miRNAs, three were reported to be downregulated in cirrhotic liver and fatty HepG2 cells (B).\cite{44,45} Error bars indicate SE. *p<0.05 in t test. **p<0.01 in t test.
The pathway analysis in this study also revealed the activation of the mTOR/Akt/PI3K and IGF-1 signaling pathways by 20–220-nm particles in BMC-CM, suggesting the possibility that humoral regulation in BMC-CM, independent of the oxygen concentration, promotes HIF-1α production.

TSG-6, which is produced in response to various inflammatory stimuli and exerts anti-inflammatory effects through various pathways (35) was also significantly upregulated upon culturing in BMC-CM in this study. It is reported that human BM-MSCs with higher mRNA expression of TSG6 show higher anti-inflammatory effects, (36) and that administration of TSG-6 suppresses liver injury in mice on a methionine choline-deficient diet through inhibition of hepatocyte apoptosis. (37) For these reasons, TSG-6 is now considered one of the most important factors responsible for inducing the immunoregulatory effects of MSCs, and a promising biomarker of their therapeutic effects. Furthermore, it is reported that TSG-6-deficient MSCs exhibit morphological changes similar to senescent or degraded MSCs and show a reduction in their proliferation and differentiation potentials, indicating that the TSG-6 produced by MSCs possibly helps to maintain the stemness of the MSCs themselves. (38)

The bone marrow (BM) and liver are closely interlinked in adult humans. (39–40) and the niche for stem/progenitor cells in the BM and liver share some similarities. MSCs are well known for their important role in establishing a niche for hematopoietic stem cells (HSCs) in BM, (41) and their possible involvement in niche formation of hepatic progenitor cells in the liver has also been reported. (42,43) This study shows that humoral factors derived from whole BM cells maintain the quality of cultured BM-MSCs. MSCs play an important role as a BM-HSC niche; conversely, BM cells may also be important in maintaining the MSC niche. Besides, the relationship between hepatic microenvironment and MSCs, which has not been well understood, may lead to the elucidation of correlation between the BM and liver mediated via MSCs.

Although it is undeniable that other factors in the BM-derived exosomes such as proteins, miRNAs, or DNAs are also involved, we identified four miRNAs that upregulate Hif1α and four miRNAs that upregulate Tsg6. The involvement of MSCs in pathophysiology of various diseases is becoming increasingly apparent, and some diseases demonstrate miRNA variations in the background. (16–18) Of the five miRNAs that upregulated Hif1α or Tsg6 in this study, three types are potentially associated with liver diseases (Fig. 5B). miR-204 and miR-92b are among the 44 primary miRNAs reportedly downregulated in liver cirrhosis in a study examining miRNA expression in surgically resected liver. (44) and miR-1247-3p and miR-92b-3p are among the 87 mature miRNAs reportedly downregulated upon fat deposition via HepG2 cells and upregulated upon liraglutide treatment. (45)

Thus, BM-derived humoral factors contain miRNAs involved in maintaining the quality of BM-MSCs through OXPHOS suppression and TSG-6 expression promotion, and some of these are also present in the hepatic microenvironment and may be downregulated in liver diseases. These results and observations indicate that BM-MSC humoral factors containing miRNAs are potentially important factors contributing to the pathogenesis of liver diseases and are mediators of the interaction between the BM and liver. Although the qualitative transformation of MSCs caused by variations in such humoral factors has unknown implications with respect to their mechanisms and significance, their application would help to improve the therapeutic effects of MSCs on liver diseases. BM-derived humoral factors containing exosomal miRNAs suppress mitochondrial OXPHOS, enhance TSG-6 expression, improve the therapeutic effects of BM-MSCs on liver injury, and are potentially involved in the pathogenesis of liver diseases. Their practical applications may help further ameliorate the quality of MSCs for regenerative therapies.

Author Contributions

TM, TT, and IS: conception and design.
TM: experiments and data analysis and interpretation.
KF, TM, and NY: provision of study, materials.
TM and TT: manuscript writing.
TT and IS: funding.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| Akt          | protein kinase B |
| BMC          | bone marrow cell |
| BMC-CM       | conditioned medium made with whole bone marrow cells |
| CCL4         | carbon tetrachloride |
| DMEM         | Dulbecco's modified Eagle medium |
| FBS          | fetal bovine serum |
| HIF-1α       | hypoxia inducible factor-1 alpha |
| HK2          | hexokinase 2 |
| HSC          | hematopoietic stem cells |
| IGF-1        | insulin-like growth factor 1 |
| MSC          | mesenchymal stem cells |
| mTOR         | mammalian target of rapamycin |
| OXPHOS       | oxidative phosphorylation |
| PBS          | phosphate buffered saline |
| PI3K         | phosphatidylinositol 3-kinase |
| ROS          | reactive oxygen species |
| Sirt         | Sirtuin |
| TSG-6        | TNF-stimulated gene 6 |
| VEGFa        | vascular endothelial growth factor A |

Conflict of Interest

No potential conflicts of interest were disclosed.

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