Changes in the Expression of Mitochondrial Morphology-Related Genes during the Differentiation of Murine Embryonic Stem Cells

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During embryonic development, cells undergo various changes in gene expression [1, 2] and signaling pathways [3]. Metabolism and intracellular organelle structures are also altered during development and differentiation [4–6]. Specifically, the organelar changes are observed during the regaining of pluripotency (also known as reprogramming) [7]. For example, Folmes et al. showed that the globular shape of mitochondria progressively changed to elongated during embryonic development from zygote to somite embryo. Accordingly, metabolic features such as pyruvate oxidation, glucose oxidation, glycolysis, and the pentose phosphate pathway (PPP) were also changed dynamically [6]. These features return to the developmental early-stage status during the reprogramming process [8]. Some of the
most dramatic changes in cells during development and differentiation occur in the mitochondria, which play essential roles in cellular processes, including energy metabolism [9], apoptosis [10], aging [11], reactive oxygen species production, calcium homeostasis, and differentiation [12].

Mitochondria continuously change their morphology through fusion and fission in response to cellular requirements, which is the crux of mitochondrial quality control. In addition, mitochondria increase their population through elongation, showing developed cristae and dense matrices [21]; this results in high oxygen consumption and ATP production for more efficient cellular activity [14, 18, 19].

In mammals, mitochondrial morphology switches between elongated tubular and fragmented globular by fusion and fission, respectively [22, 23]. Mitochondrial fusion is mediated by the dynamin family GTPases, such as mitofusin (MFN) 1, MFN2, and optic atrophy 1 (OPA1) [24–26]. Although the exact fusion mechanism has yet to be defined, MFN1 and MFN2 form a dimer that inserts itself into the mitochondrial inner membrane, whereas OPA1 contains a GTPase domain, hydrophobic heptad repeat (HR) domain, and transmembrane domain [24, 29]. MFN1 and MFN2 play similar roles in mitochondrial fusion and thus can functionally replace each other [24, 29]. MFN1 and MFN2 form a dimer that inserts itself into the mitochondrial inner membrane [27, 28]. MFN1, MFN2, and OPA1 contain a GTPase domain, hydrophobic heptad repeat (HR) domain, and transmembrane domain [24, 29]. MFN1 and MFN2 play similar roles in mitochondrial fusion and thus can functionally replace each other and form homotypic or heterotypic dimers [25, 30].

In contrast, the major proteins related to mitochondrial fission are FIS1 [31–33] and dynamin-related protein 1 (DNM1L, also called DRP1) [34–36]. DNM1L is mainly located in the cytosol and recruited to the outer membrane of the mitochondria where it induces fission [30]. FIS1 is located in the outer mitochondrial membrane and is closely related to DNM1L [32, 33]. DNM1L can interact with other mitochondrial fission proteins, including mitochondrial fission factor (MFF) and FIS1. Interestingly, a recent study suggested that DNM1L can interact with the fusion protein MFN and facilitate MFN-mediated fission [30]. Although many studies have evaluated the fusion and fission of mitochondria, the mechanisms and signaling pathways that determine mitochondrial dynamics are still unclear. Activation of the mammalian target of rapamycin (mTOR) pathway by the withdrawal of LIF induced mouse ESC differentiation with suppression of pluripotent genes such as Klf4, Oct4, and Nanog [37]. When pluripotent stem cells differentiate, they require more energy to meet the demands of their newly acquired functions [6].

Accordingly, mitochondria undergo dynamic remodeling during differentiation, and thus, mitochondrial morphology and metabolism are changed. Therefore, we hypothesized that expression of mitochondrial fusion- and fission-related genes may be changed toward a certain direction during the spontaneous differentiation of ESCs. Here, we quantified the expression levels of the fusion-related genes Mfn1, Mfn2, and Opa1 and the fission-related genes Fis1 and Dnm1L during the differentiation of murine ESCs. Here, we investigated these genes to determine whether they could be used as an index of the extent of differentiation and changes in mitochondrial morphology.

2. Materials and Methods

All methods used in this study were carried out in accordance with animal care and use guidelines, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University.

2.1. Cell Cultures. Mouse ESCs (E14tg2a) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured on culture dishes layered with inactivated MEFs in an ESC medium, consisting of Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 15% fetal bovine serum (FBS; HyClone), 1× Penicillin/Streptomycin/Glutamine (P/S/G; Gibco), 0.1 mM nonessential amino acids (NEAAs; Gibco), and 1 mM β-mercaptoethanol (Gibco) with 1000 U/mL leukemia inhibitory factor (ESGRO, Chemicon International). MEFs were cultured in culture dishes coated with 0.15% porcine gelatin (Sigma, St. Louis, MO, USA) in MEF medium consisting of DMEM (Gibco) supplemented with 15% FBS (HyClone), 1x P/S/G (Gibco), 0.1 mM NEAAs (Gibco), and 1 mM β-mercaptoethanol (Gibco). NSCs were cultured in culture dishes coated with 0.15% porcine gelatin (Sigma) in NSM medium consisting of DMEM:Nutrient Mixture F-12 (Gibco), 0.5 mg/mL bovine serum albumin (BSA; Sigma), 1% N2 supplement (Gibco), 1x NEAAs (Gibco), 1x P/S/G (Gibco), 10 ng/mL basic fibroblast growth factor (bFGF; R&D systems), and 10 ng/mL epidermal growth factor (EGF; Gibco). All cell lines were incubated at 37°C in an atmosphere of 5% CO2 and maintained on tissue culture dishes (Corning, Amsterdam, The Netherlands).

2.2. In Vitro Differentiation. Pluripotent ESCs maintain stemness in the presence of LIF via the JAK-STAT3 pathway. However, when this pathway is inhibited in response to LIF withdrawal, pluripotent ESCs randomly differentiate into endodermal, ectodermal, and mesodermal cells. Cherepkova et al. showed that LIF withdrawal induced mouse ESC differentiation via the activation of the mTOR pathway [37]. Based on the differentiation protocol, we have established an ESC differentiation protocol that has been customized for our laboratory conditions. In the preplating process, ESCs cultured with feeder cells were dissociated by trypsin-EDTA (0.25%) (Gibco) and transferred to a 0.15% gelatin-coated dish and
incubated at 37°C in an atmosphere of 5% CO₂ for 2 h to remove the feeder cells. Because the feeder cells attached to the gelatin-coated dish much earlier than ESCs, the supernatant of the culture mostly contains ESCs without contamination of feeder cells. The supernatant was transferred to another gelatin-coated dish and used as the differentiation experiment. For in vitro differentiation, 1 × 10⁵ ESCs were seeded in 100 mm cell culture dishes coated with 0.15% porcine gelatin (Sigma) with MEF medium. The medium was refreshed every day for 15 days of differentiation. The cells were collected by scraping for experimental analysis.

2.3. RNA Isolation and qRT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The cDNA was then synthesized from 1 mg total RNA using SuperScript III Reverse Transcriptase (Invitrogen) and oligo(dT)20 primer according to the manufacturer’s instructions. qPCR was performed in duplicate with Power SYBR Green Master Mix (Takara, Shiga, Japan), and results were analyzed using 1.43 (NIH) software. Protein expression levels were normalized to those of Actb.

2.4. Western Blot Analysis. Total cells were lysed using RIPA buffer (Thermo Fisher) according to the manufacturer’s instructions. Cell lysates (20 μg protein) were separated on NuPAGE 4–12% Bis-Tris Gels (Invitrogen) and transferred to polyvinyl difluoride membranes. The membranes were blocked using a blocking solution containing 5% skim milk powder and 0.05% Tween 20 in phosphate-buffered saline (PBS). Primary antibodies used in these studies were as follows: anti-OCT4 (rabbit, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nanog (rabbit, 1:1000; Abcam, Cambridge, UK), anti-MFN2 (mouse, 1:1000; Abcam), anti-DRP1 (rabbit, 1:1000; Millipore), anti-MFN1 (mouse, 1:1000; Abcam), anti-FIS1 (mouse, 1:500; Abcam), and anti-β-actin (mouse, 1:10000; Sigma). The membranes were incubated with these antibodies overnight at 4°C. Secondary antibodies were conjugated with anti-mouse IgG-peroxidase (1:10000; Sigma), anti-goat IgG-horseradish peroxidase (HRP), and anti-rabbit IgG-HRP (1:10000; Santa Cruz Biotechnology), and the membranes were incubated with these antibodies for 90 min at room temperature.

Antigens were detected using Pierce ECL Western Blotting chemiluminescent substrate (Thermo Fisher), according to the manufacturer’s instructions. Blots were then exposed to X-ray film for development and stripped for reuse of the membranes. Anti-β-actin antibody (mouse, 1:10000; Sigma) was used as a control. Densitometry of the bands for the proteins and their loading controls was performed using ImageJ 1.43 (NIH) software. Protein expression levels were normalized to those of Actb.

2.5. Immunocytochemistry. For immunocytochemistry, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were washed with PBS and then treated with PBS containing 3% bovine serum albumin and 0.03% Triton X-100 for 30 min at room temperature. The cells were then incubated with the following primary antibodies: anti-OCT4 (1:500; Santa Cruz Biotechnology), anti-Nanog (1:200; Abcam), anti-β3-tubulin (TUJ1; 1:500; R&D), anti-SMA (1:200; Abcam), anti-SOX17 (1:300; R&D), and anti-TOM20 (1:200; Santa Cruz Biotechnology). Fluorescently labeled (Alexa Fluor 488 or 647; Abcam) secondary antibodies were used according to the manufacturer’s specifications. Images for anti-TOM20 staining were obtained with a confocal microscope (Zeiss).

2.6. Electron Microscopy. For transmission electron microscopy (TEM) experiments, the samples were fixed in 4% paraformaldehyde (Sigma) and 2.5% glutaraldehyde (Sigma) in 0.1 M phosphate (Sigma) buffer for 24 h. After washing in 0.1 M phosphate buffer, the samples were postfixed for 1 h in 1% osmium tetroxide (Sigma) prepared in the same buffer. The samples were dehydrated with a graded series of ethyl alcohol concentrations, embedded in Epon 812, and polymerized at 60°C for 3 days. Ultrathin sections (60–70 nm) were obtained using an ultramicrotome (Leica Ultracut UCT), collected on grids (200 mesh), and examined under a TEM (JEM 1010) operating at 60 kV, and images were recorded by a charge-coupled device camera (SC1000; Gatan).

### Table 1: Primer sets used for quantitative RT-PCR.

| Gene   | Forward                        | Reverse                        |
|--------|--------------------------------|--------------------------------|
| Mfn1   | 5′-CATGGGCATCATATGCCGTGGG-3′   | 5′-TCTCCACTGTCGCGGTAG-3′       |
| Mfn2   | 5′-CAAGTGTCGGCTCTGAAGG-3′     | 5′-GAACCTCCTTGGCACACAG-3′      |
| Opa1   | 5′-CAAGGATTACAGAGGGTGATAC-3′  | 5′-CAGTGAGCATCACCCCTAC-3′      |
| Fis1   | 5′-CATCGTGCCTGGAGAGC-3′       | 5′-GCCGTTGTCAACAGCAGTAC-3′     |
| Dnm1L  | 5′-GAAGTTGAACGAGATAAGGG-3′    | 5′-TGGCATCGTTCATCATGCTAC-3′    |
| Oct4   | 5′-GATCTGTCGAGCCAAGGCAAG-3′   | 5′-CTCCATGACCTGGTGGT-3′        |
| Nanog  | 5′-CTTTCACCTATTTAGTTGC-3′     | 5′-CGAAGCCGCTTGTGGTGCAC-3′     |
| T      | 5′-CGGGTGCTGAAGTAAATGT-3′     | 5′-CGTCACTGTCGAGATATG-3′       |
| Actb   | 5′-CGCCATGGATGACGATATCG-3′    | 5′-CGAAGCCGCTTGTGGTGCAC-3′     |
2.7. Mitochondrial Length Analysis. The images from electron microscopy were analyzed and measured by the ImageJ 1.43 (NIH) software for calculating the maximum (Max)/minimum/(Min) ratio of mitochondrial length. At least over fifty mitochondria were measured and analyzed per sample to obtain data.

2.8. Statistical Analysis. All experiments were performed in triplicate, and data are presented as mean ± standard error of mean (SEM). Differences were assessed using one-way ANOVA with Tukey’s honestly significant difference (HSD) post hoc or Fisher’s least significant different (LSD) post hoc for multiple comparisons appropriately, and differences with p values of less than 0.05 were considered significant.

3. Results

3.1. Changes in Pluripotency- and Tissue-Specific Markers during the Differentiation of Mouse ESCs. To examine changes in gene expression during the differentiation of ESCs, we randomly differentiated mouse ESCs by the withdrawal of LIF without feeder cells for 0, 3, 6, 9, 12, and 15 days (Figure 1(a)). Dome-like colonies of undifferentiated ESCs became flat and showed changes in morphology. First, we checked whether ESCs were properly differentiated in vitro after 15 days in differentiation medium. Immunocytochemistry analysis showed that Oct4 and Nanog, which were expressed in undifferentiated ESCs, were silenced at day 15 after differentiation. Differentiation markers, neuron-specific class III β-tubulin (TuJ1), smooth muscle actin (SMA), and SRY-box 17 (Sox17) for ectodermal, mesodermal, and endodermal cells, respectively, were not detectable in undifferentiated ESCs at day 0 but were detected after differentiation at day 15, indicating that ESCs lost pluripotency and were differentiated into various cell types, including all three germ layers (Figure 1(b)).

Next, we evaluated the expression of pluripotency and differentiation markers by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Figures 1(c) and 1(d)). The expression levels of the pluripotency markers Oct4 (F = 34.18, p < 0.001) and Nanog (F = 1927.49, p < 0.001) were gradually decreased as ESCs were differentiated (Figure 1(c)). In contrast, early mesoderm marker T (also known as Brachyury) (one-way ANOVA with Tukey’s HSD, F = 455.71, p < 0.001) expression was increased until day 6 postdifferentiation and then downregulated afterward (Figure 1(d)), indicating that early mesoderm cells appeared at day 6 after differentiation and then differentiated further. Taken together, our findings demonstrated that ESCs differentiated gradually and lost pluripotency over 15 days upon LIF withdrawal from the ESC medium.

3.2. Changes in Mitochondrial Morphology during the Differentiation of Mouse ESCs. Mitochondrial morphology was expected to change from fragmented to elongated shapes during the differentiation of ESCs. At first, we investigated the mitochondrial biogenesis by immunostaining using antibodies targeting translocase of the outer membrane 20 (TOM20), which is in the outer mitochondrial membrane during the differentiation of ESCs. In ESCs, mitochondria (green dots in Supplementary Fig. 1) were evenly distributed in the cytoplasm. However, the number of green dots had been increased since the differentiation of ESCs (Supplementary Fig. 1). Next, we observed the mitochondrial morphologies accurately by electron microscopic analysis during the differentiation of ESCs (Figure 2(a)). Undifferentiated ESCs had primarily globular mitochondria with immature cristae, and this morphology was maintained until day 3 of differentiation. From days 6 to 15, mitochondria were gradually elongated and showed mature cristae (Figure 2(a)). We measured the Max and Min axes of mitochondria (one-way ANOVA with Tukey’s HSD, Max: F = 24.25, p < 0.001; Min: F = 31.87, p < 0.001) to quantify mitochondrial length (Figures 2(b) and 2(c)) including mitochondrial perimeter and area (Supplementary Fig. 2a-b). The Max/Min ratios of mitochondria (one-way ANOVA with Tukey’s HSD, F = 27.10, p < 0.001) were 1.51, 1.66, 3.65, 3.92, 4.30, and 6.87 on days 0, 3, 6, 9, 12, and 15, respectively (Figure 2(d)). These data clearly demonstrated that the globular and immature mitochondria in ESCs became elongated and showed developed mature cristae after the spontaneous differentiation.

3.3. Changes in Mitochondrial Morphology-Related Genes during the Differentiation of Mouse ESCs. Because mitochondria were elongated during the differentiation of ESCs, we predicted that fusion-related genes would be upregulated and fission-related genes would be downregulated during this differentiation. Thus, we examined mitochondrial morphology-related genes by qRT-PCR analysis. Unexpectedly, the fusion-related gene Mfn1 (one-way ANOVA with Tukey’s HSD, F = 604.95, p < 0.001) was progressively downregulated, whereas Mfn2 (one-way ANOVA with Tukey’s HSD, F = 11.10, p < 0.001) and Opa1 (one-way ANOVA with Tukey’s HSD, F = 85.53, p < 0.001) expression patterns fluctuated at day 3 postdifferentiation (Figure 3(a)). Next, we evaluated the expression of two fission-related genes, Dnm1L and Fis1 (Figure 3(b)); consistent with the reduced mitochondrial fission, which resulted in enhanced mitochondrial elongation, the expression of Dnm1L (one-way ANOVA with Tukey’s HSD, F = 385.64, p < 0.001) decreased as mouse ESCs underwent spontaneous differentiation (Figure 3(b)). However, Fis1 (one-way ANOVA with Tukey’s HSD, F = 2.57, p = 0.083) expression did not decrease but rather increased after slight downregulation at the beginning of differentiation (Figure 3(b)). This result supported previous findings that FIS1 is less related to mitochondrial fission than DNML1 and functions to recruit DNML1 to the mitochondria [38, 39]. Collectively, our results showed that Mfn1 and Dnm1L mRNA expression levels reflected mitochondrial elongation with ESC differentiation.

Accordingly, we next investigated the levels of Mfn2 and DNML1 proteins by western blotting in ESCs (day 0) and in differentiated cells on days 0, 3, 6, 9, 12, and 15 (Figure 3(d)). As a control for ESC spontaneous differentiation, the expression of the pluripotency marker OCT4 gradually decreased during differentiation and was almost undetectable at day 15. Mfn2 was expressed at a low level in ESCs but showed a dramatic increase in expression upon
Figure 1: *In vitro* differentiation and characterization of ESCs. (a) Phase-contrast images of ESCs and differentiating cells on days 0, 3, 6, 9, 12, and 15. Scale bars = 200 μm. (b) Immunofluorescence images of the pluripotency markers Oct4 and Nanog and differentiation markers for ectodermal (Tuj1), mesodermal (SMA), and endodermal (Sox17) cells in undifferentiated ESCs and ESC-derived differentiated cells on days 0 and 15. Nuclei was counterstained with DAPI. Scale bars = 100 μm. (c, d) Quantitative RT-PCR analysis of ESCs and differentiating cells on days 0, 3, 6, 9, 12, and 15 (D0, D3, D6, D9, D12, and D15). Data are presented as mean ± SEM for n = 3 independent experiments. (c) Pluripotent marker Oct4 and Nanog expression on days 0 to 15. (d) Differentiation marker T expression from days 0 to 15. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus D0.
spontaneous differentiation. Furthermore, MFN2 protein levels showed a fluctuating expression pattern during differentiation, similar to the qRT-PCR data (Figures 3(a) and 3(d)). DNM1L protein levels decreased gradually during the differentiation of ESCs, similar to the result of qRT-PCR analysis (Figures 3(b) and 3(d)). Thus, we observed similar changes in the mRNA and protein levels for these two targets.

3.4. Establishment of Indexes Representing the Extent of Differentiation. Given that many genes involved in mitochondrial fusion and fission are not correlated with mitochondrial morphology, we next attempted to find an index to represent mitochondrial morphology. Based on the qRT-PCR data, we analyzed the ratios between three fusion- and two fission-related genes during the differentiation of ESCs. A total of 6 combinatorial ratios for three fusion- and two fission-related genes were analyzed (Figure 3(c)). One of these, the Mfn2/Dnm1L ratio (one-way ANOVA with Tukey’s HSD, $F = 31.24, p < 0.001$), was interesting because it was similar to the mitochondrial length Max/Min ratio (Supplementary Fig. 3a). Next, we confirmed the protein expression level in the Mfn2/Dnm1L ratio (one-way ANOVA with Tukey’s HSD, $F = 25.56, p < 0.001$) (Figure 3(e), Supplementary Fig. 4). Indeed, the Mfn2/Dnm1L ratio was also similar to the mRNA expression and mitochondrial length Max/Min ratio. Thus, we concluded that the mitochondrial changes during ESC spontaneous differentiation could be reflected by the progressive increase in the Mfn2/Dnm1L ratio.

3.5. Mfn2/Dnm1L Index Represented Mitochondrial Morphology according to the Extent of Differentiation. To investigate whether this index was applicable to other cell types, we compared this ratio among ESCs, neural stem cells (NSCs), and mouse embryonic fibroblasts (MEFs). We chose these cell types because ESCs are undifferentiated, NSCs are less specialized, and MEFs are differentiated. Only ESCs express high levels of Oct4 (one-way ANOVA with Fisher’s LSD, $F = 33.18, p < 0.001$) and Nanog (one-way ANOVA with Fisher’s LSD, $F = 2051.34, p < 0.001$) (Figure 4(b)). Although the mRNA expression level of the mitochondrial fusion-related gene Mfn2 (one-way ANOVA with Fisher’s LSD, $F = 7.12, p < 0.05$) was slightly changed in these cell types, the mRNA expression level of the mitochondrial
Figure 3: Continued.
fission-related gene Dnm1L (one-way ANOVA with Fisher’s LSD, $F = 804.07, p < 0.001$) was gradually decreased in NSCs and MEFs (Figures 4(c) and 4(d)), consistent with the observed changes in mitochondrial morphology (Figure 4(a)). Next, we applied the Mfn2/Dnm1L ratio (one-way ANOVA with Fisher’s LSD, $F = 50.42, p < 0.001$) to these cell types, with adjustment to a ratio of 1.0 for ESCs. Interestingly, the adjusted Mfn2/Dnm1L ratios were 1.0, 2.97, and 3.86 in ESCs, NSCs, and MEFs, respectively. Next, we also confirmed Mfn2 and Dnm1L protein expression (Figure 4(f)). As expected, the Dnm1L protein expression levels were decreased in NSCs and MEFs, compared with those in ESCs. Interestingly, the Mfn2 protein expression levels were gradually increased in NSCs and MEFs. The Mfn2/Dnm1L protein expression ratios (one-way ANOVA with Fisher’s LSD, $F = 19660.5, p < 0.001$) were 0.16, 2.94, and 4.61 in ESCs, NSCs, and MEFs, respectively. Thus, we concluded that the Mfn2/Dnm1L ratio at the mRNA and protein expression levels corresponded to the length of mitochondria (Figures 4(e)–4(g)).

4. Discussion

In this study, we investigated the dynamics of mitochondrial morphology-related genes, which are responsible for mitochondrial fusion and fission, during the differentiation of mouse ESCs. Given that differentiated cells contain elongated mitochondria and ESCs contain globular mitochondria [9], we expected to observe increases in the expression of mitochondrial fusion-related genes (Mfn1, Mfn2, and Opa1) and decreases in the expression of fission-related genes (Fis1 and Dnm1L).

This is because several reports have shown that the overexpression of fusion-related genes such as Mfn1/Mfn2 [25] and Opa1 [40] induces mitochondrial elongation in MEFs. In this context, the overexpression of fission-related genes such as Drp1 [41] and Fis1 [42] induces mitochondrial fragmentation in MEFs and HeLa cells, respectively. However, there were only minor changes in the expression levels of Mfn1 and Opa1 during differentiation, and changes in the expression of Fis1 were opposite to the expected results. Actually, FIS1 has been reported to have a weaker effect on mitochondrial fission than DNM1L in MEFs but not in HeLa [39, 41, 42] possibly due to the different mechanisms of mitochondrial morphology regulation in humans and mice. A loss-of-function experiment in a previous study showed that Mfn1 or Mfn2 deficiency results in fragmented mitochondrial morphology and loss of Mfn2 has more dramatic effects than loss of Mfn1 [25]. Moreover, OPA1, processed for mitochondrial fusion by protease isoenzymes and OMA1, might not reflect the morphology of mitochondria [43]. This is because various proteins affect the processing of OPA1 protein for its function in the mitochondrial fusion.

Next, we aimed to identify a special index that gradually increased during ESC spontaneous differentiation. We found that a gradual increase in the Mfn2/Dnm1L ratio was closely related to mitochondrial elongation during the elapsed time after the differentiation of ESCs. Additionally, we conducted correlation analysis of both Mfn2/Dnm1L and Mfn1/Fis1 (one-way ANOVA with Tukey’s HSD, $F = 463.54, p < 0.001$) ratios with mitochondrial length, because these ratios had consistent patterns like increase or decrease, respectively. Interestingly, the Mfn2/Dnm1L ($R^2 = 0.8874$) ratio pattern was more correlated with mitochondrial length than the Mfn1/Fis1 ($R^2 = 0.4149$) ratio (Supplementary Fig. 3a-b). Furthermore, we found that this index could be applied to other cell types, including NSCs and MEFs. More specialized cells showed higher Mfn2/Dnm1L ratios than ESCs.

There were some limitations to our research. Firstly, technologies such as high-content imaging and machine learning were recently developed for the analysis of the shapes of mitochondria [44]. This technical method allows the analysis of the mitochondria by further subdividing the shape of the mitochondria, e.g., fragmented, rods, networks,
and large/round. However, the functions of the genes that affect the dynamic of mitochondria have yet to be identified and therefore cannot explain how mitochondria form a network [9]. Therefore, we focused on classifying the mitochondria in just two categories, i.e., fragmented and elongated. For further study, the combination of high-throughput gene screening by using RNA sequencing for sorting the gene set related to the mitochondrial dynamics will be necessary; the high-resolution imaging system with the machine learning depends on the mitochondrial morphology according to cell types. Then, we will ultimately be able to understand the various phenomena related to the shape of mitochondria and how we can regulate it. Second, some cell types at final stages of differentiation such as erythropoietic cells [45, 46] and hepatocytes [47] have fragmented mitochondria. If ESCs were differentiated by LIF withdrawal, various cell types will be observed among differentiated cell population [37, 48], including erythropoietic cells and hepatocytes. The fragmentation of mitochondria related to the specific role of cells cannot be limited to gene expression, which controls the dynamic of mitochondria. Otherwise, we showed the mitochondrial perimeter and area in Supplementary Fig. 2a-b. The error bars of these gradually expanded according to the passing of time. It means that there are various cell types which have various morphologies of mitochondria in the differentiating day 15.

**Figure 4:** Mfn2/Dnm1L ratios in ESCs, NSCs, and MEFs. (a) Electron microscopic images of mitochondria in undifferentiated ESCs, NSCs, and MEFs. Yellow dotted lines represent mitochondrial morphologies. Scale bars = 0.5 μm. Quantitative RT-PCR analysis of (b) pluripotent-, (c) mitochondrial fusion-, and (d) mitochondrial fission-related genes in ESCs, NSCs, and MEFs. Gene expression levels were normalized to those of Actb. (e) Mfn2/Dnm1L gene expression ratios in ESCs, NSCs, and MEFs. (f) Western blotting for the expression of DNM1L and MFN2 in ESCs, NSCs, and MEFs. β-Actin was used as a control for other proteins. (g) MFN2/DNM1L protein ratios in ESCs, NSCs, and MEFs. All data are presented as mean ± SEM for n = 3 independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 versus D0.
Thus, the tendency of gene expression patterns can only be interpreted as the indexes that predict the degree of differentiation and shape of mitochondria, because of the random differentiation process (i.e., no lineage-specific differentiation). Thus, our findings suggested that this ratio could also be used as an index for mitochondrial morphology during differentiation.

**Data Availability**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethical Approval**

All methods used in this study were carried out in accordance with animal care and use guidelines, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University.

**Conflicts of Interest**

The authors declare that they have no conflict of interest.

**Authors’ Contributions**

JEL, BJS, KH, and JTD wrote the main manuscript text and designed the study. JEL, BJS, YJH, and MJH performed experiments and analyzed the data. JEL, BJS, HS, and JWL performed data analysis. All authors reviewed the manuscript. Jeong Eon Lee and Bong Jong Seo contributed equally to this work.

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**Supplementary Materials**

Supplementary Figure 1: expression pattern of TOM20 on days 0, 3, 6, 9, 12, and 15 after differentiation of ESCs. Green dots represent mitochondria. Nuclei was counterstained with DAPI. Scale bars = 10 μm.

Supplementary Figure 2: (a) the mitochondrial perimeter (μm) in ESCs on differentiating days 0, 3, 6, 9, 12, and 15. (b) The mitochondrial area (μm²) in ESCs on differentiating days 0, 3, 6, 9, 12, and 15. Data are presented as mean ± SEM for n = 50 independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 versus D0.

Supplementary Figure 3: (a) correlation analysis between the Mfn2/Dnm1L ratio and the maximal length of mitochondria normalized to ESCs (D0). (b) Correlation analysis between the Mfn1/Fis1 ratio and the maximal length of mitochondria normalized to ESCs (D0).

Supplementary Figure 4: (a) normalized protein level of DNM1L protein on days 0, 3, 6, 9, 12, and 15 after differentiation of ESCs. (b) Normalized protein level of MFN2 protein on days 0, 3, 6, 9, 12, and 15 after differentiation of ESCs. Protein expression levels were normalized to those of Actb. All data are presented as mean ± SEM for n = 3 independent experiments. ***p < 0.001 versus D0. (Supplementary Materials)

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