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Differentiation of embryonic stem cells into inner ear vestibular hair cells using vestibular cell derived-conditioned medium

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ABSTRACT

Vestibular hair cells (V–HCs) in the inner ear have important roles and various functions. When V–HCs are damaged, crippling symptoms, such as vertigo, visual field oscillation, and imbalance, are often seen. Recently, several studies have reported differentiation of embryonic stem (ES) cells, as pluripotent stem cells, to HCs, though a method for producing V–HCs has yet to be established. In the present study, we used vestibular cell conditioned medium (V-CM) and effectively induced ES cells to differentiate into V–HCs. Expressions of V-HC-related markers (*Math1*, *Myosin6*, *Brn3c*, *Dnah5*) were significantly increased in ES cells cultured in V-CM for 2 weeks, while those were not observed in ES cells cultured without V-CM. On the other hand, the cochlear HC-related marker *Lmod3* was either not detected or detected only faintly in those cells when cultured in V-CM. Our results demonstrate that V-CM has an ability to specifically induce differentiation of ES cells into V–HCs.

1. Introduction

Inner ear hair cells (HCs) residing in the cochlea have important roles related to hearing [1–3], while those in the vestibular system are involved with balance [4–6]. Notably, vestibular HCs (V–HCs) are essential for such functions as gaze stabilization, balance, and gravity perception [7,8]. When V–HCs are damaged, crippling symptoms, including vertigo, visual field oscillation, and imbalance, are often seen [9,10]. Although cochlear HCs in mammals cannot be regenerated, V–HCs are considered to retain a renewal capability [11–14], and various methods for their spontaneous regeneration have been shown in *in vitro* and *in vivo* findings related to gene therapy [15–17]. However, an effective mechanism for regeneration of V–HCs remains to be elucidated.

Embryonic stem (ES) and induced-pluripotent stem (iPS) cells are useful for examining the process of differentiation to a specific cell type, and several methods for inducing differentiation into HCs have been recently reported [18–21]. We previously showed *in vitro* induction of HC-like cells from mouse ES cells using conditioned medium obtained from an ST2 stromal cell line, termed the HIST2 method [22], and those results indicated that various factors secreted from cells induced differentiation into HCs. In addition, we found that regulation of *Math1* (HC differentiation-related gene) in combination with the HIST2 method induced HC-like cells more efficiently than each alone [23]. However, a method targeting V–HCs with cell-conditioned medium has not been reported.

In the present study, we attempted differentiation of ES cells into V–HCs using the differentiation-inducing activity of supernatant obtained from cultures of vestibular cells (VCs) isolated from the inner ears of postnatal mice. Conditioned medium (CM) from cultured VSs (V-CM) successfully promoted differentiation of ES cells into V-HC-like cells. Our results demonstrate important roles of V-CM for ES cell differentiation into V–HCs.

2. Materials and methods

2.1. Cells

Mouse *Math1-GFP* ES cells, a kind gift from Dr. K. Muguruma (RIKEN CDB, Kobe, Japan), were established, and carried the modified

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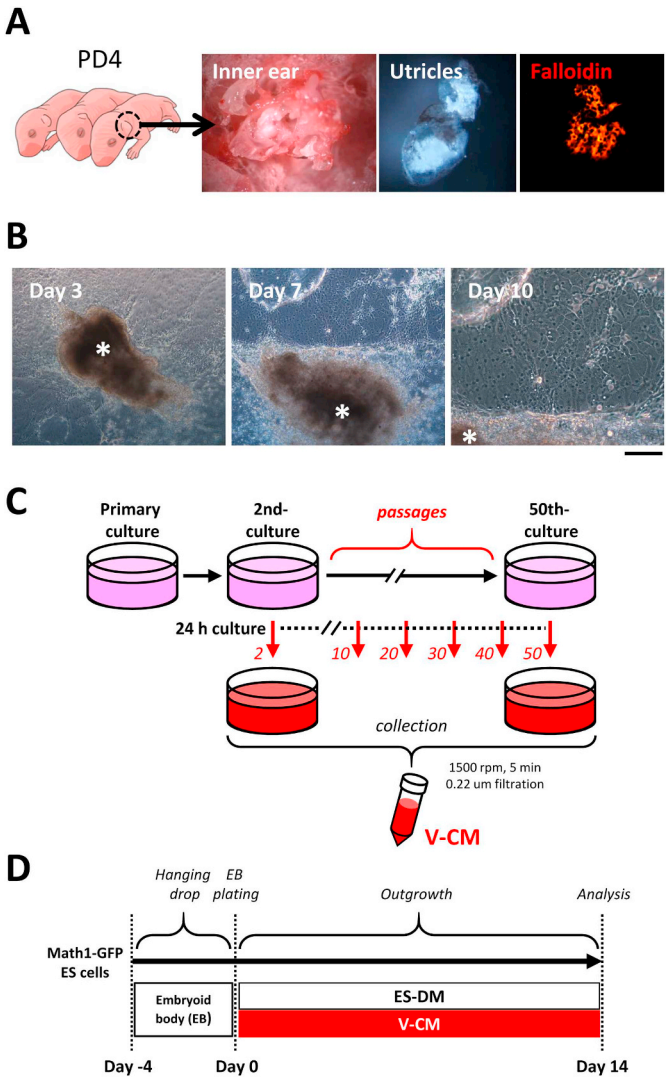

Fig. 1. Preparation of vestibular cells (VCs) and conditioned medium, and *in vitro* differentiation procedure. (A) Murine utricles were isolated from the inner ears of postnatal day 4 (PD4) C57BL/6 mice using a microdissection method. Isolated utricles were confirmed using RITC-labeled phalloidin. (B) Vestibular cells (VCs) showing outgrowth from utricles were obtained and cultured in ES-DM. Asterisks indicate utricle attachment to the dish. Scale bar = 50 μm. (C) Conditioned medium (CM) obtained from VCs cultured in ES-DM for 24 h was collected, then centrifuged and filtrated, and used as VC conditioned medium (V-CM). (D) *In vitro* hair cell differentiation procedure.

GFP gene (Venus) driven by a human β-globin promoter (−37 to +21) and *Math1* enhancer (mouse genomic sequences containing Enhancers A and B) [24]. *Math1-GFP* ES cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% FBS (GIBCO, Invitrogen, Carlsbad, CA), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (GIBCO), 1 mM sodium pyruvate (Wako), and 1000 U/ml of leukemia inhibitory factor (LIF; Wako) on gelatin-coated dishes without feeder cells.

2.2. Preparation and cultivation of vestibular cells (VCs)

All animal studies were conducted under an institutional protocol approved by Nara Medical University. Murine utricles were isolated from the inner ears of postnatal day 4 (PD4) C57BL/6 mice using a microdissection method previously reported [25,26]. The utricle covered with cartilage was exposed by fenestration of the overlying cartilaginous plate, then carefully pulled from the bony preparation (Fig. 1A). Successfully isolated utricles were divided into 2 samples, one of which was confirmed using RITC-labeled phalloidin (Molecular Probes, Invitrogen) (Fig. 1A), while the other was cultured in ES cell medium without LIF (ES-DM), then proliferating cells were used as vestibular cells (VCs) and maintained in ES-DM (Fig. 1B).

2.3. Preparation of conditioned medium (CM)

The supernatants were accumulatively collected from the 24 h-cultures of the 2nd, 10th, 20th, 30th, 40th, 50th-passaged VCs. The samples were then centrifuged and filtrated through a 0.22-μm syringe membrane filter (Miilipore, Billerica, MA), and finally prepared as VC-conditioned medium (V-CM) (Fig. 1C).

2.4. In vitro hair cell differentiation

Differentiation of undifferentiated *Math1-GFP* ES cells into vestibular hair cells was performed using the procedure shown in Fig. 1D. *Math1-GFP* ES cells were dissociated by trypsin and cultured in hanging drops to form embryoid bodies (EBs) at a density of 3000 cells/20 μl ES-DM [27]. After 4 days, EBs were collected and plated in 35-mm gelatin-coated plastic dishes (10 EBs per dish). They were allowed to attach as outgrowth cultures for a period of 14 days in ES-DM or V-CM, with half of the culture medium changed to new medium every 2 days.

Differentiation of *Math1-GFP* ES cells was monitored using GFP fluorescence, with detection performed using fluorescence microscopy (BZ-X710, Keyence, Osaka, Japan) and flow cytometry (FACS Calibur, BD Bioscience) methods.

2.5. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA (1 μg) was extracted from cultured cells using TRIzol reagent (Invitrogen), then reverse transcription and qPCR were performed using a SYBR PrimeScript RT-PCR kit II (TaKaRa Bio Inc., Osaka, Japan), according to the manufacturer's instructions. Primers used for qPCR were purchased from TaKaRa Bio Inc. and are shown in Supplementary Table S1. Relative quantitation was performed using the ΔΔCT method. Data were normalized to the expression of *β-actin* or *RPLP0* as an endogenous control, and are presented as relative gene expression.

2.6. Immunocytochemistry

Immunofluorescence analysis was performed using a standard protocol. Briefly, cells were fixed in 4% paraformaldehyde, then cellular membranes were permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA (TPBS). All primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used at the following dilutions; anti-GFP (1:100), anti-Brn3c (1:100), anti-myosin6

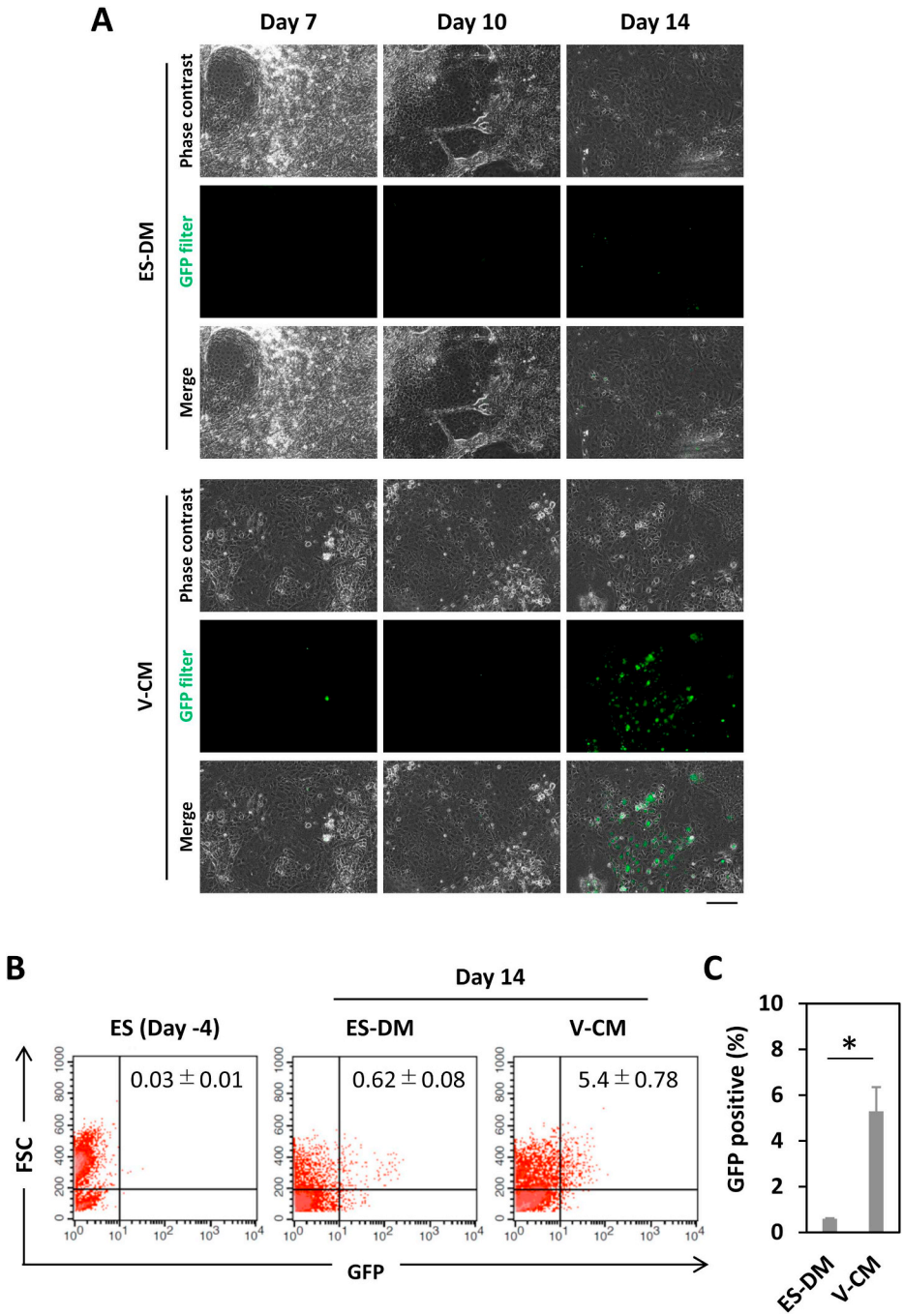

Fig. 2. Cell morphology and GFP expression of *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM. (A) Cell morphology and GFP expression of *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM for 2 weeks. Scale bar = 100 μm. (B) Flow cytometrical analysis of *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM for 2 weeks. (C) GFP positivity of *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM for 2 weeks.

(1:100), Lmod3 (1:100), and Dnah5 (1:100). Following incubation overnight at 4 °C and washing 3 times with TPBS, AlexaFluor 488 or 546 conjugated anti-goat, anti-rabbit, or anti-mouse secondary antibodies (Molecular Probes, Invitrogen) were used to detect primary antibodies. All nuclei were stained with DAPI (Dojin, Kumamoto, Japan). After incubation for 1 h at room temperature and washing with TPBS 3 times, fluorescence was detected using fluorescence microscopy (BZ-X710).

2.7. Statistical analysis

Data are expressed as the mean ± SD of 5 independent experiments. Statistical significance was tested using Student's *t*-test, with a p value < 0.05 considered to indicate significance.

3. Results

3.1. Cell morphology and GFP expression of Math1-GFP ES cells cultured in V-CM

Four-day EBs, formed in hanging drop cultures of *Math1-GFP* ES cells, were cultured in ES-DM or V-CM for 14 days. The morphologies of EB outgrowths cultured in ES-DM or V-CM for 2 weeks were not clearly different in phase contrast observation findings (Fig. 2A, phase

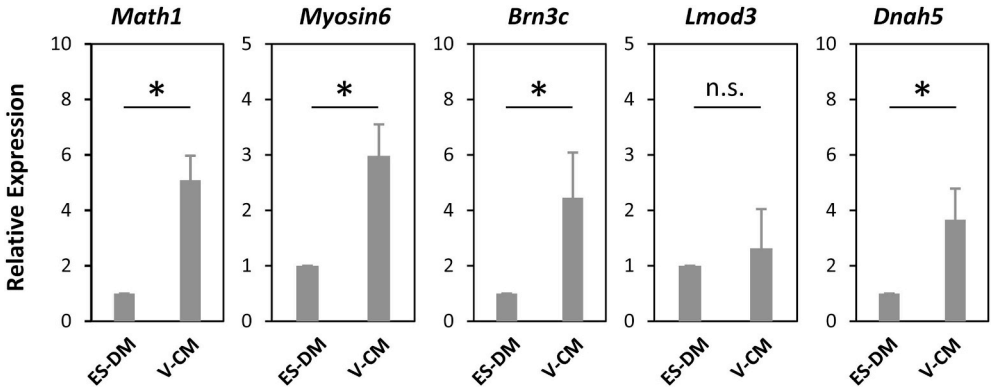

Fig. 3. Gene expression analysis of hair cell-related markers in *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM. Gene expressions of *Math1*, *Myosin6*, and *Brn3c*, (HC-related markers), *Lmod3* (cochlear HC-related marker), and *Dnah5* (vestibular HC-related marker) by *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM for 2 weeks were examined using real-time RT-PCR. Values were normalized to that of *β-actin* expression, used as an endogenous control. $^{*}p < 0.05$.

contrast). However, in observations conducted using a GFP filter, GFP-positive cells were detected in the outgrowths cultured in V-CM but not in those cultured in ES-DM (Fig. 2A, GFP filter). Moreover, flow cytometry findings showed an increase in *Math1*-derived GFP positivity of 5.4 ± 0.78% in the *Math1-GFP* ES cells cultured in V-CM, while that was not detected in those cultured in ES-DM (0.62 ± 0.08%) (Fig. 2B and C). These results indicated that V-CM effectively induced differentiation of ES cells into cells positive for *Math1*.

3.2. Gene expression analysis of hair cell-related markers

Total RNA was extracted from EB outgrowths cultured in ES-DM or V-CM for 2 weeks, then gene expressions of hair cell (HC)-related markers were examined using a real-time RT-PCR method. A recent study demonstrated that *Lmod3* and *Dnah5* are markers of cochlear and vestibular HCs, respectively [28]. Thus, we used those, as well as the HC-related markers *Math1*, *Myosin6*, and *Brn3c* [20,23,29], and examined differentiation of ES cells into HCs (Fig. 3). Expressions of *Math1*, *Myosin6*, *Brn3c*, and *Dnah5* were significantly increased in EB outgrowths cultured in V-CM as compared to those in ES-DM. However, there was no significant difference regarding the gene expression of *Lmod3* observed in EB outgrowths cultured in either medium formulation. These results suggested that V-CM promotes differentiation of ES cells into HC-like cells, seemingly with a preference for vestibular HC-like cells.

3.3. Immunocytochemical analysis of hair cell-related markers

We also used an immunocytochemical method to examine the expressions of HC-related markers in EB outgrowths cultured for 2 weeks in ES-DM or V-CM. In EB outgrowths cultured in ES-DM, no GFP expression nor that of any of the examined HC-related markers, including *Myosin6*, *Brn3c*, *Dnah5*, *and Lmod3*, was observed (Fig. 4, ES-DM). On the other hand, GFP-positive cells were clearly observed in EB outgrowths cultured in V-CM and those showed simultaneous expression of *Myosin6*, *Brn3c*, and *Dnah5* (Fig. 4, V-CM). In contrast to the distinct emergence of *Dnah5*-immunopositive cells, *Lmod3*-immunopositive cells were either not detected or detected only faintly in EB outgrowths cultured in V-CM.

4. Discussion

The bHLH class transcription factor *Math1* is known to be expressed during development of the nervous system in multiple domains, including not only the vestibular and auditory systems, but also the dorsal neural tube and external germinal layer of the cerebellum [30–34]. In the present study, *Math1*-derived GFP positive cells were detected in EB outgrowths cultured in V-CM for 2 weeks (Fig. 2), then HC-related markers were analyzed in order to examine whether GFP positive cells are the inner ear hair cells-like cells.

In analyses of real time RT-PCR results, V-CM was found to promote expression of the HC-related markers *Math1*, *Myosin6*, *Brn3c*, and *Dnah5,* but not that of *Lmod3* (Fig. 3). Furthermore, our preliminary investigation revealed expressions of markers of both immature and mature HCs, including *Nestin*, which is a well-known marker of neural stem cells and immature HC progenitors [35–37], as well as *Grp* and *Espin*, markers of mature HCs [28,38,39]. Additionally, expressions of *Nestin*, *Grp*, and *Espin*, markers of mature HCs, were upregulated in the present ES cells cultured in V-CM (Supplementary Fig. S1), whereas those were not detected in EB outgrowths cultured in ES-DM. These results suggested that V-CM has a differentiation-inducing activity (DIA) on the 4-day EB-derived cells into vestibular HC-like cells.

In addition to the gene expression experimental results, immunocytochemical analysis findings also revealed distinct emergence of cells immunopositive for *Dnah5*, a vestibular HC marker, as well as those positive for *Math1*, *Myosin6*, and *Brn3c* (HC marker) in EB outgrowths cultured with V-CM, whereas those were not seen in cells cultured with ES-DM (Fig. 4). On the other hand, *Lmod3* (cochlear HC marker) immunopositivity was not different between the V-CM and ES-DM cultures.

In the present study, supernatants were accumulatively collected from the 24 h-cultures of the 2nd, 10th, 20th, 30th, 40th, 50th-passaged VCs. Analyses of those showed that V-CM had a DIA on the 4-day EBs that showed differentiation toward vestibular HCs, though the DIA of the supernatants from the respective passaged cultures was not separately examined. It is considered that precise analysis of each supernatant sample from passaged cultures might lead to selection of CM possessing a DIA with high efficiency.

We also examined murine skin fibroblast-derived CM (F-CM) to determine the DIA. No or very few GFP-positive cells were observed in EB-outgrowths of *Math1-GFP* ES cells cultured with F-CM, which was confirmed by flow cytometry findings (0.23 ± 0.09 positivity). Furthermore, the expression levels of *Math1*, *Myosin6*, *Brn3c*, and *Dnah5* in EB-outgrowths cultured with F-CM were similar to those obtained by culturing in ES-DM (Supplementary Fig. S2), while gene expression and immunocytochemical analysis results unexpectedly revealed *Lmod3* expression to some extent (Supplementary Fig. S2 and Fig. S3, respectively). Recent studies have reported the expression of *Lmod3* in skeletal and cardiac muscle tissues [40,41], which was shown to be related to thin filament disorganization and nemaline myopathy [42]. In the present experiments, *Lmod3* expression was not associated with *Math1*-derived GFP expression in cultures with F-CM, suggesting differentiation into non-cochlear *Lmod3*-expressing cells.

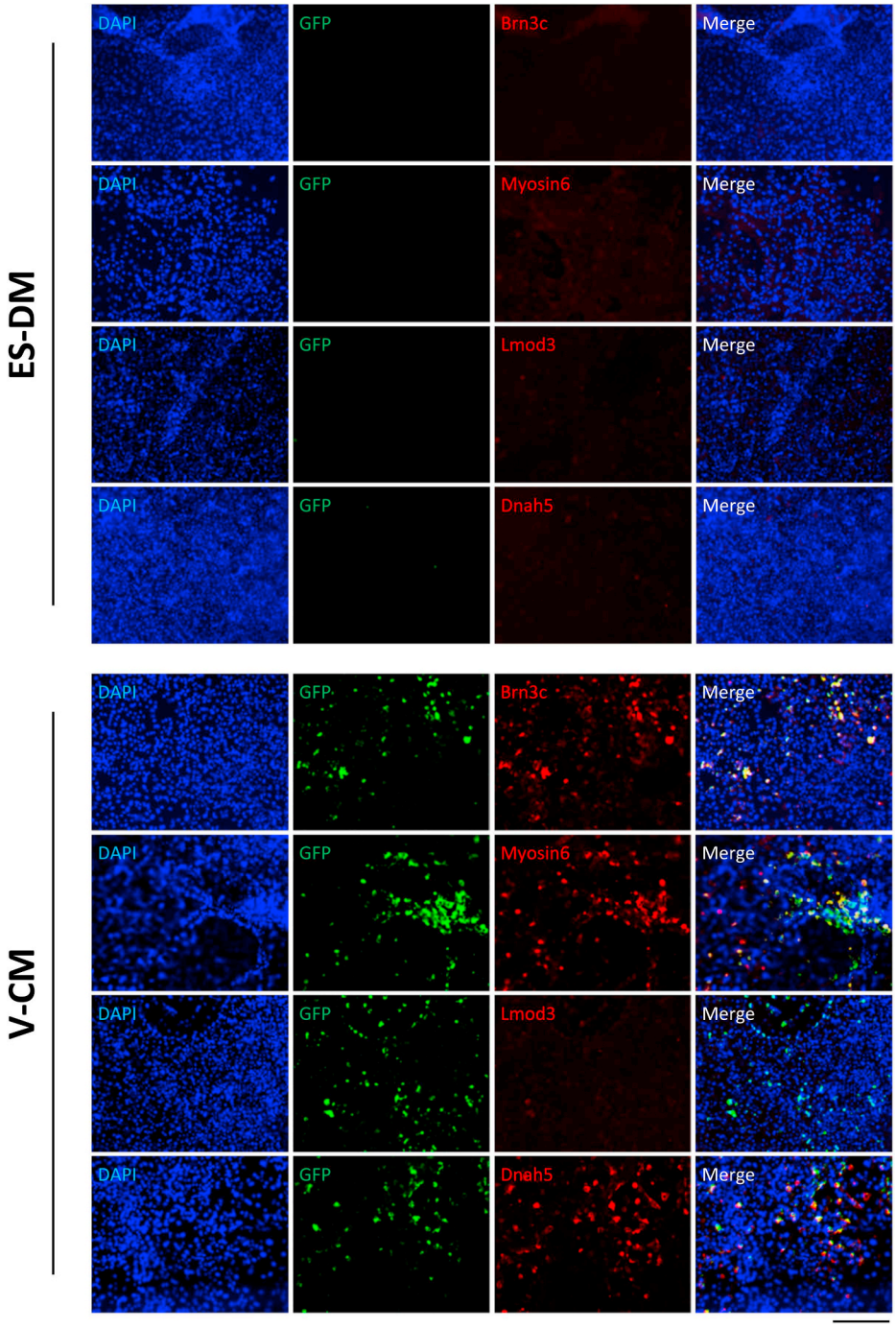

Fig. 4. Immunocytochemical analysis of hair cell-related markers expressed by *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM. The expressions of HC-related markers in *Math1-GFP* ES cell-derived EBs cultured in ES-DM or V-CM for 2 weeks were examined with an immunocytochemical method. Neither HC-related markers nor *Math1*-derived GFP were detected in EB outgrowths cultured in ES-DM, whereas most of the *Math1*-derived GFP positive cells in EB outgrowths cultured in V-CM simultaneously expressed Myosin6, Brn3c, and Dnah5. On the other hand, no Lmod3-immunopositive cells were detected in EB outgrowths cultured in V-CM, while *Math1*-derived GFP positive cells were detected. Scale bar = 100 μm.

Authors' contributions

M.S., Y.O., and M.Y. conceived and designed the experiments. M.S., Y.O., and N.K. performed the experiments and statistical analysis. N.K. M.M., and T.K. contributed to preparation of the manuscript. M.S., Y.O., and M.Y. wrote the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of interest

None of the authors have conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100649.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100649.

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