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In vitro differentiation of mouse embryonic stem cells into inner ear hair cell-like cells using stromal cell conditioned medium

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Hearing loss is mainly caused by loss of sensory hair cells (HCs) in the organ of Corti or cochlea. Although embryonic stem (ES) cells are a promising source for cell therapy, little is known about the efficient generation of HC-like cells from ES cells. In the present study, we developed a single-medium culture method for growing embryoid bodies (EBs), in which conditioned medium (CM) from cultures of ST2 stromal cells (ST2-CM) was used for 14-day cultures of 4-day EBs. At the end of the 14-day cultures, up to 20% of the cells in EB outgrowths expressed HC-related markers, including Math1 (also known as Atoh1), myosin6, myosin7a, calretinin, α9AchR and Brn3c (also known as Pou4f3), and also showed formation of stereocilia-like structures. Further, we found that these cells were incorporated into the developing inner ear after transplantation into chick embryos. The present inner ear HC induction method using ST2-CM (HIST2 method) is quite simple and highly efficient to obtain ES-derived HC-like cells with a relatively short cultivation time.

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Inner ear hair cells (HCs) function as the primary transducers for perception of sound and balance, while defects in their formation or loss result in sensory deficits.[1] In mammals, once HCs are lost, they are not regenerated.[2,3] Therefore, their reproduction requires various medical strategies (e.g., gene therapy and cell therapy).[4–6] Gene therapy using overexpression of the transcription factor Math1 (also known as Atoh1) via viral infection was reported to generate new HCs and substantially improve hearing thresholds in adult deaf guinea pigs.[7,8] Furthermore, recent advances in the understanding of molecular mechanisms related to HC formation and stem cell technology have raised the possibility that HC-like cells might be generated from some stem cell sources using new methods,[9,10] and then applied in translational therapies for individuals with hearing loss.[11]

Although there are several candidate cell sources for cell therapy, including neural stem cells (NSCs) from adult brains,[5] mesenchymal stem cells from bone marrow,[12] and olfactory precursor cells,[13] embryonic stem (ES) cells and induced-pluripotent stem cells are particularly promising because of their ability for self-renewal and pluripotency.[14,15] A number of recent studies have shown that ES cells differentiate into a variety of different cell types *in vitro*.[16,17] However, only a few reports have documented *in vitro* differentiation of ES cells into HCs.[18–21] Furthermore, the methods employed in those studies were rather time consuming and complicated by multiple steps such as formation of embryoid bodies (EBs), expansion of nestin-positive cells, and exposure to several cytokines, while the results provided very small numbers of cells expressing HC-related markers.[20,21]

In the present study, we developed a simple single-medium culture method for growing EBs, in which conditioned medium (CM) from ST2 stromal cells (ST2-CM) was used for 14-day cultures. After the end of culturing with ST2-CM, ~20% of cells in EB outgrowths expressed HC-related markers, including Math1, myosin6, myosin7a, calretinin, α9AchR, and Brn3c (also known as Pou4f3), while formation of stereocilia-like structures was also seen. We further found that those cells were incorporated into the developing inner ear after transplantation into chick embryos. This inner ear hair cell induction method using ST2-CM (HIST2 method) is quite simple and highly efficient to obtain ES-derived HC-like cells with a relatively short period of cultivation.

Results

Directed neural differentiation of ES cells by stromal cells. EB formation was performed using a hanging drop method with ES-M without leukemia inhibitory factor (LIF) for 4 days. After EB formation, 4-day EBs were cultured with various differentiated media (Table 1) for

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Keywords: embryonic stem cells; inner ear; hair cells; differentiation; conditioned medium; stromal cells
Abbreviations: CM, conditioned medium; EBs, embryoid bodies; ES, embryonic stem; HCs, hair cells; HIST2 method, hair cell induction method using ST2-CM; iPS, induced-pluripotent stem; NSCs, neural stem cells; PA6-CM, conditioned medium from PA6 stromal cells; SCs, supporting cells; SEM, scanning electron microscope; ST2-CM, conditioned medium from ST2 stromal cells
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Table 1 Differentiation medium types

	FBS	KOS	N2, B27	bFGF	PA6[a]	ST2[a]
ES-M	+	−	−	−	−	−
KOS-M	−	+	−	−	−	−
N2-M	−	−	+	−	−	−
N2-M + bFGF	−	−	+	+	−	−
PA6-CM	+	−	−	−	+	−
ST2-CM	+	−	−	−	−	+

Abbreviations: CM, conditioned medium; FBS, fetal bovine serum; PA6-CM, conditioned medium from PA6 stromal cells; ST2-CM, conditioned medium from ST2 stromal cells
[a]Medium conditioned with feeder cells for 48 h

2 weeks (Figure 1a). In cultures with ES-M or KOS-M, the appearance of EB outgrowths was rather heterogeneous and often included beating cells (Figures 1b1 and b2). EB outgrowths in cultures with N2-M had a 'network' appearance (Figures 1b3), while those in cultures with N2-M containing bFGF showed a homogenous sheet-like appearance (Figures 1b4). In the cultures with PA6-CM, large numbers of cellular clusters were observed around the originally attached EBs (Figures 1b5). The characteristic morphology observed in cultures with ST2-CM was prominent neurite-like outgrowths emerging from the aggregates (Figures 1b6).

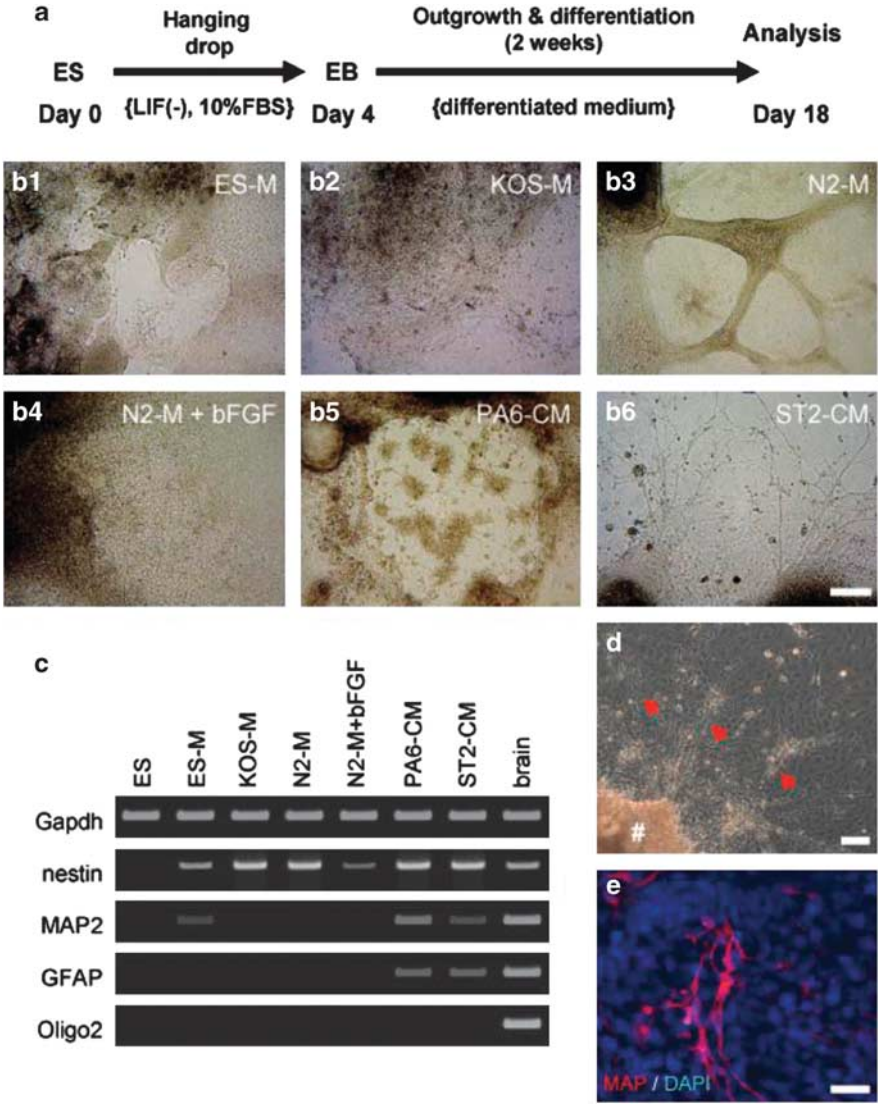

Figure 1 Directed neural differentiation of ES cells by stromal cells. (**a**) Schedule of differentiation. Following EB formation, EBs were cultured with various differentiated media (see Table 1) for 2 weeks. (**b**) Differentiation of EBs was induced using various media, as listed in Table 1. The photographs indicate EB outgrowths after 2 weeks of cultivation: (**b**-1) ES-M, (**b**-2) KOS-M, (**b**-3) N2-M, (**b**-4) N2-M + bFGF, (**b**-5) PA6-CM, (**b**-6) ST2-CM. Scale bar = 100 μm. (**c**) Analysis of expressions neural markers (nestin, MAP2, GFAP, Oligo2) in EB outgrowths after cultivation in various differentiation media for 2 weeks. Samples from adult mouse brain tissues served as a positive control. (**d**) EB outgrowths cultured with ST2-CM possessed neurite-like structures. The symbol '#' and red arrows indicate EB body and neurite-like extension, respectively. Scale bar = 100 μm. (**e**) The neurite-like extensions were immuno-positive for MAP2. Scale bar = 20 μm

After 2 weeks of cultivation of EBs, neural differentiation was assessed using a semi-quantitative RT-PCR method (Figure 1c). RNA samples were prepared from undifferentiated ES cells and EB outgrowths, and the expressions of nestin, MAP2, GFAP, and Oligo2 were examined. Samples from adult mouse brain tissues served as a positive control. No neural expression was observed in undifferentiated ES cells, whereas nestin, a marker for NSCs,[22] was expressed in all of the samples from EB outgrowths. The expression of MAP2, a marker for neurons, was observed only in samples cultured with ES-M, PA6-CM, or ST2-CM, while GFAP, a marker of glial astrocytes, was expressed only in those cultured with PA6-CM or ST2-CM. The expression of Oligo2 was not observed in any of the samples. The neurite-like structures seen in EB outgrowths cultured with ST2-CM (Figure 1d) were immuno-positive for MAP2 (Figure 1e).

Gene expressions of HC-related markers in the EB outgrowths. Gene expressions of the HC-related markers Brn3c, myosin6, myosin7a, and α9AchR[21] were analyzed using a semi-quantitative RT-PCR method after 2-week cultivations of EBs with various media (Figure 2a). Samples taken from the inner ear of an embryonic day 14 (E14) mouse were served as a positive control. No expression was observed in undifferentiated ES cells. The expression of myosin7a was found to be prevalent in all EB outgrowths. However, expressions of the other three genes were only observed in EB outgrowths cultured with ST2-CM and not in those with PA6-CM. To confirm that the induction of such gene expression specific for HCs was because of CM from the culture of ST2 cells, EBs were cultured with media containing graded amounts of ST2-CM (Figure 2b). The expressions of nestin and myosin7a occurred independently of ST2-CM, whereas those of myosin6, Math1, Brn3c, and α9AchR were dependent on ST2-CM and required a concentration greater than 50%. These results suggest that ST2-CM contains effective humoral factors to promote the expression of HC-related markers in EB outgrowths.

Immunocytochemical analysis of specific markers for HCs in EB outgrowths. After 2 weeks of differentiation of EBs in ES-M or ST2-CM, EB outgrowths were analyzed for HC-related markers using an immunohistochemical method (Figure 3). In EB outgrowths cultured with ES-M, cells immuno-positive for Brn3c, Math1, myosin6, and α9AchR were seen at very low frequencies (less than 2% for each), while myosin7a- and calretinin-immuno-positive cells comprised 29.1 ± 2.1% and 10.3 ± 1.1%, respectively (Figure 3a and Supplementary Figure S1A). In contrast, remarkably increased numbers of immuno-positive cells were detected in EB outgrowths cultured with ST2-CM. The percentages of immuno-positive cells for the investigated markers were as follows: Brn3c, 21.2 ± 2.2%; myosin7a, 51.3 ± 3.2%; calretinin, 38.4 ± 4.1%; Math1, 24.8 ± 3.5%; myosin6, 31.2 ± 2.9%; and α9AchR, 27.3 ± 4.1%. ST2-CM dramatically promoted the emergence of cells immuno-positive for HC-related markers in the EB outgrowths. In contrast, cells immuno-positive for HC-related markers were scarcely detected in EB outgrowths cultured with ES-M + KOS, N2-M, N2-M + bFGF, or PA6-CM, similar to those cultured with ES-M.

The simultaneous expression of Brn3c, myosin6, and calretinin with Math1 was examined in EB outgrowths cultured with ES-M and complete ST2-CM (100%) using an immunocytochemical method. Among Math1-positive cells generated in cultures with ES-M, the rates of co-expression with Brn3c, myosin6, and calretinin were 17.2 ± 2.1%, 26.3 ± 1.3%, and 16.1 ± 2.4%, respectively (Supplementary Figure S1B). However, in cultures with ST2-CM, more than 80% of Math1-positive cells expressed Brn3c (84.3 ± 4.2%), myosin6 (87.2 ± 3.5%), and calretinin (84.3 ± 5.1%), indicating that most of the Math1-positive cells induced in ST2-CM simultaneously expressed those markers (Figure 3b and Supplementary Figure S1B). These results also strongly suggest that ST2-CM has an ability to preferentially promote the generation of cells expressing HC-related markers in EB outgrowths.

Stereocilia-like structures formed in HC-like cells. A characteristic cytomorphology of HCs is the presence of stereocilia.[23] EB outgrowths allowed to differentiate in ST2-CM for 2 weeks were examined using phalloidin staining, immunocytochemistry for Espin,[24] Brn3c, acetylated tubulin, and scanning electron microscope (SEM). Some of the cells had phalloidin-labeled protrusions (Figure 4a). Highly reminiscent or V-shaped actin-rich hair bundles were recognized

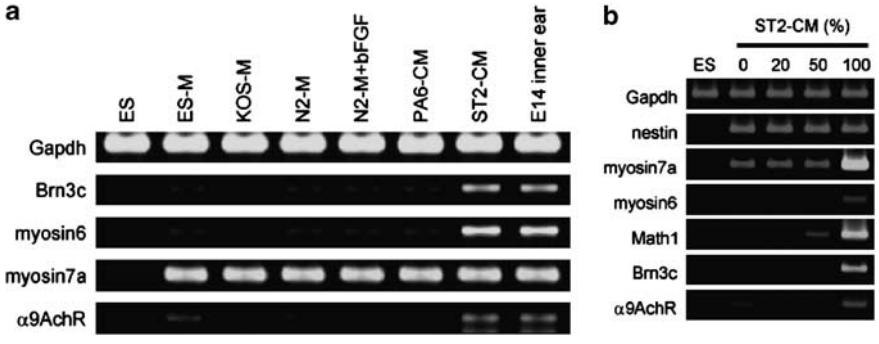

Figure 2 Gene expressions of HC-related markers in EB outgrowths. (**a**) Gene expressions of HC-related markers (Brn3c, myosin 6, myosin 7a, and α9AchR) in EB outgrowths cultured with various media were analyzed using a RT-PCR method. Samples from day 14 embryonic (E14) mouse inner ears served as a positive control. (**b**) Analysis of gene expression of HC-related markers was performed with various concentrations of ST2-CM

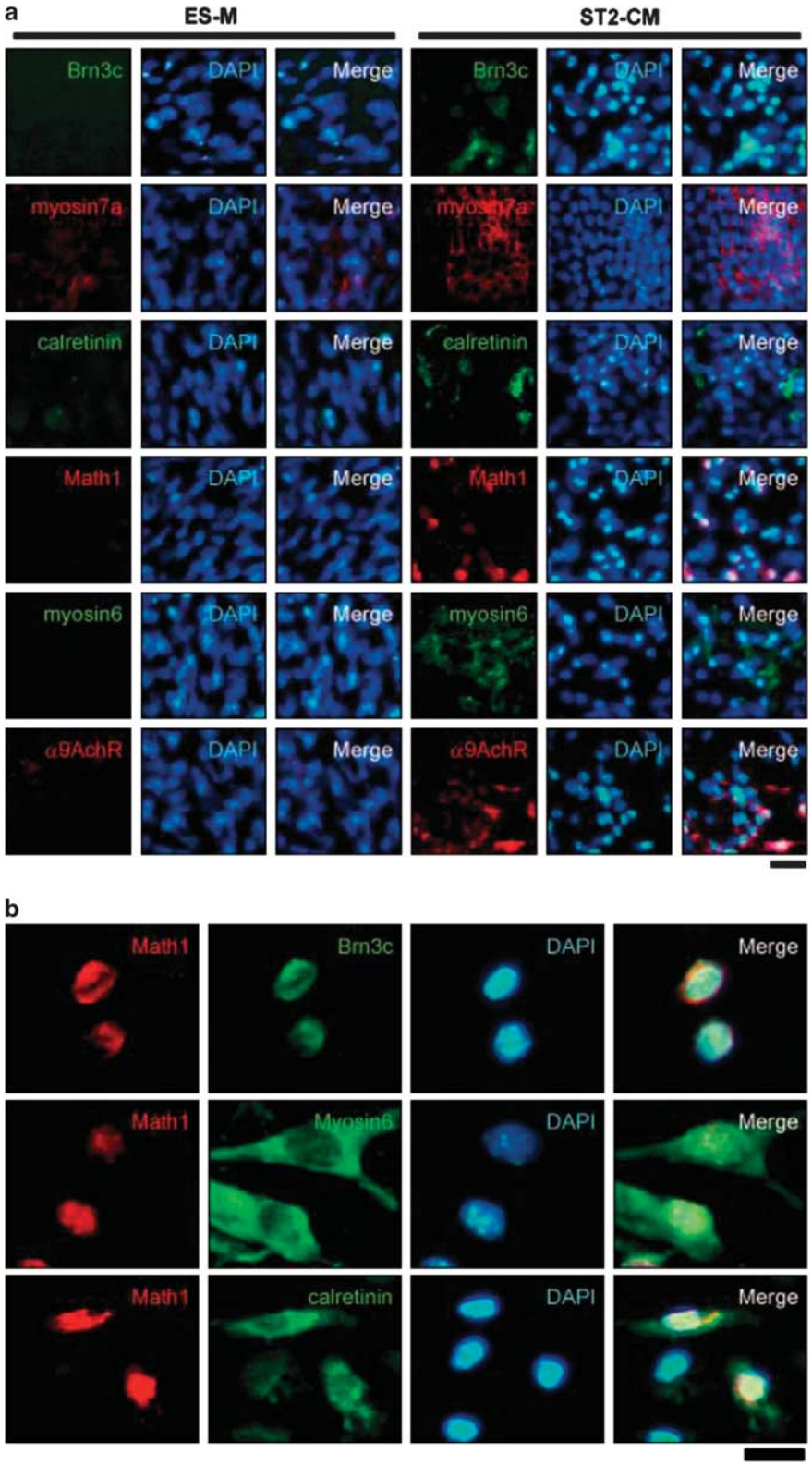

Figure 3 Immunocytochemical analysis of specific markers for HCs in EB outgrowths. (**a**) After 2 weeks of differentiation of EBs in ES-M or ST2-CM, EB outgrowths were analyzed for HC-related markers (Brn3c, myosin7a, calretinin, Math1, myosin6, and α9AchR) using an immunohistochemical method. ST2-CM dramatically induced the emergence of immuno-positive cells for those markers in EB outgrowths. Scale bar = 20 μm. (**b**) Simultaneous expressions of Brn3c, myosin6, and calretinin with Math1 were examined in EB outgrowths cultured with ST2-CM using an immunocytochemical method. Scale bar = 10 μm

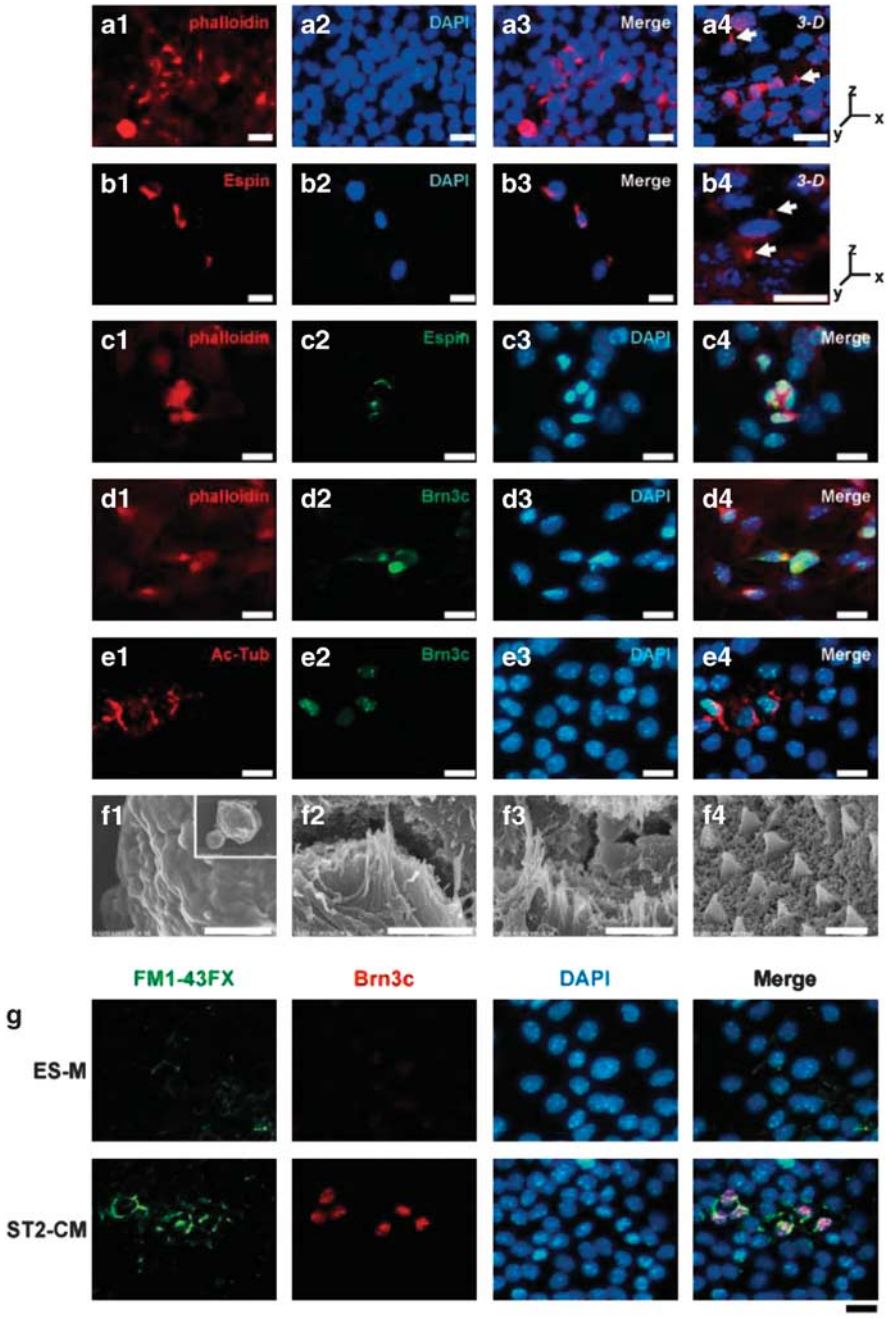

Figure 4 Stereocilia-like structures formed in HC-like cells. (**a–f**) EB outgrowths cultured with ST2-CM for 2 weeks were examined with phalloidin staining and immunostaining for Espin, Brn3c, acetylated tubulin as well as with a SEM. (**a**) Some cells showed phalloidin-labeled protrusions, highly reminiscent of actin-rich hair bundles (arrow). Scale bars = 10 μm. (**b**) Espin-immuno-positive cells were detected, and the immuno-positivity was found in protrusions at the cell rim (arrow). Scale bars = 10 μm. (**c**) Espin-immuno-positive cells (**c**-2, green) were simultaneously stained for phalloidin (**c**-1, red). Scale bars = 10 μm. (**d**) Brn3c-immuno-positive cells (**d**-2, green) were simultaneously stained for phalloidin (**d**-1, red). Scale bars = 10 μm. (**e**) Acetylated-tubulin-immuno-positivity (**e**-1, red) was found in Brn3c-immuno-positive cells (**e**-2, green). Scale bars = 10 μm. (**f**) Distinct stereocilia-like structures were confirmed by SEM analysis. Shown are undifferentiated ES cells (**f**-1, low magnification in inset), ES cells differentiated with ST2-CM (**f**-2 and **f**-3), and inner ear HCs from the organ of Corti of postnatal mice (day 1) (**f**-4). Scale bars = 3 μm (**f**-1), 2 μm (**f**-2), 3 μm (**f**-3), 5 μm (**f**-4). (**g**) The mechanotransduction channel function was examined based on rapid permeation of FM1-43FX dye (green), then immunostaining with Brn3c (red) was performed. Scale bars = 20 μm

along the rim of the pear-shaped cell bodies (Figures 4a4, arrow). Furthermore, cells immuno-positive for Espin were also detected (Figure 4b) and phalloidin-labeled cells were positive for Espin (Figure 4c), while Brn3c-immuno-positive cells were simultaneously immuno-positive for phalloidin and acetylated tubulin (Figures 4d and e, respectively). On the other hand, the EB outgrowths cultured with ES-M + KOS, N2-M, N2-M + bFGF, or PA6-CM did not contain the cells immuno-positive for phalloidin, Brn3c, Espin, or acetylated tubulin.

Distinct stereocilia-like structures were examined using SEM observations. Undifferentiated ES cells did not have any of those structures (Figures 4f1), while some cells in the EB outgrowths in ST2-CM possessed stereocilia bundles (Figures 4f2 and f3). An SEM image of HCs from the organ of Corti of postnatal mice (day 1) is shown in Figures 4f4. The stereocilia bundle s were 2–3 μm long and similar to those seen in ES cells cultured in ST2-CM.[25]

The potential of functional mechanotransduction channels were examined using FM1-43FX dye, which is used to identify the onset of transduction channel function by virtue of rapid permeation in developing HCs.[13,26] A marked number of FM1-43FX-positive cells (21.3 ± 1.6%) were identified in EB growths cultured with ST2-CM (Figure 4g, ST2-CM and Table 2). In contrast, FM1-43FX positivity was seen at a very low frequency (0.52 ± 0.1%) in EB outgrowths cultured with ES-M (Figure 4g, ES-M). In addition, immunostaining with Brn3c was performed following FM1-43FX dye treatment and fixation. Nearly all FM1-43FX-positive cells were Brn3c-immuno-positive (Table 2), indicating that ST2-CM dramatically induced the emergence of HC-like cells with potentially functional mechanotransduction channels.

Table 2 FM1-43FX uptake in HC-like cells

	ES-M	ST2-CM
FM1-43FX+ (%)[a]	0.52 ± 0.1	21.3 ± 1.6
Brn3c+ (%)[b]	0.45 ± 0.1	19.2 ± 2.1
FM1-43FX+/Brn3c+ (%)[c]	17.2 ± 0.2	92.3 ± 4.5

Abbreviation: ST2-CM, conditioned medium from ST2 stromal cells
[a]The percentages were quantified using FM1-43FX-positive cells and nuclei stained by DAPI ($n = 15$)
[b]The percentages were quantified using Brn3c-immuno-positive cells and nuclei stained by DAPI ($n = 15$)
[c]Brn3c immunostaining was performed after FM1-43FX dye treatment and fixation. The percentages of FM1-43FX-positive cells in Brn3c-immuno-positive cells are shown ($n = 15$)

Recapitulated developmental processes in induction of HC-like cells from ES cells with ST2-CM. To investigate the process of generation of HC-like cells from ES cells, we examined the time-course of gene expression related to neural differentiation and HC development in 18-day cultures, which encompassed 4 days for EB formation and the subsequent 14 days for EB outgrowths in ST2-CM. Nestin was expressed in the 4-day EBs, while myosin7a expression was seen by day 7, that of myosin 6 and Math1 was seen by day 11, and Brn3c and α9AchR were finally expressed by day 18 (Figure 5a).

Similarly to the results of gene expression, an immunocytochemical examination to detect Brn3c-positive cells in EB outgrowths grown in ST2-CM revealed that Brn3c-positive cells were scarcely seen during the first week of the EB cultures, while their numbers were drastically increased by day 14 (Figure 5b). In immunocytochemical analysis, cells immuno-positive for $p27^{kip1}$, a marker for supporting cells (SCs),[27] as well as cells immuno-positive for calretinin, a marker for HCs, were seen in EB outgrowths cultured with ST2-CM for 18 days (Figure 5c). Three expression patterns were observed, which were $p27^{kip1}$-positive and calretinin-negative cells (Figure 5c, short arrow), $p27^{kip1}$-negative and calretinin-positive cells (arrowhead), and $p27^{kip1}$-positive and calretinin-positive cells (long arrow).

Transplantation of cells from EB outgrowths containing HC-like cells into chick embryos. To examine whether HC-like cells are integrated in living animals by grafting, we transplanted cells from EB outgrowths grown in ST2-CM into

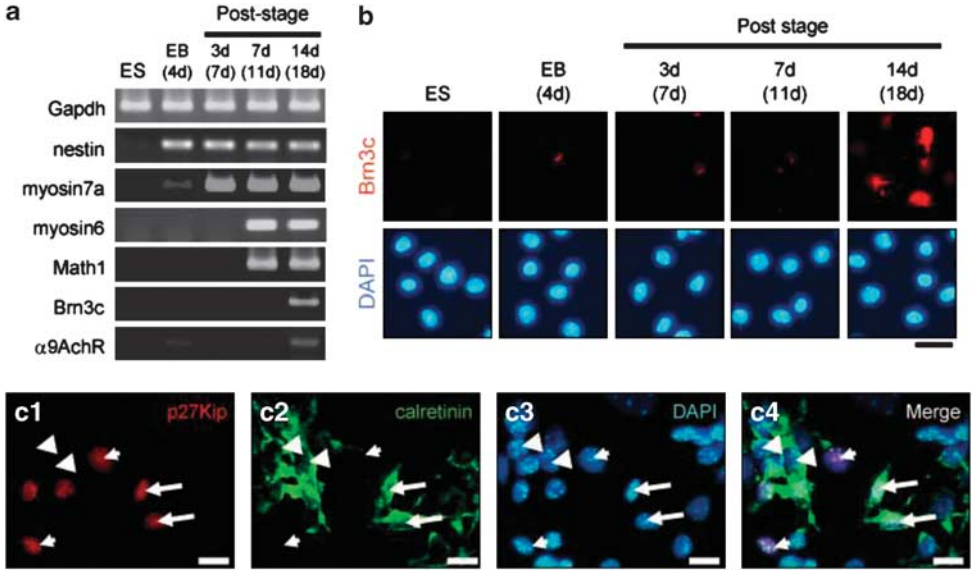

Figure 5 Recapitulated developmental processes in induction of HC-like cells from ES cells with ST2-CM. (**a**) Time-course analysis of gene expressions for HC-related markers using RT-PCR method. (**b**) Time-course analysis of immunocytochemical expression of Brn3c. Scale bar = 10 μm. (**c**) Immunocytochemical analysis of $p27^{kip1}$ and calretinin in EB outgrowths cultured with ST2-CM for 14 days. Three expression patterns were observed, which were $p27^{kip1}$-positive and calretinin-negative cells (short arrow), $p27^{kip1}$-negative and calretinin-positive cells (arrowhead), and $p27^{kip1}$-positive and calretinin-positive cells (long arrow). Scale bars = 10 μm

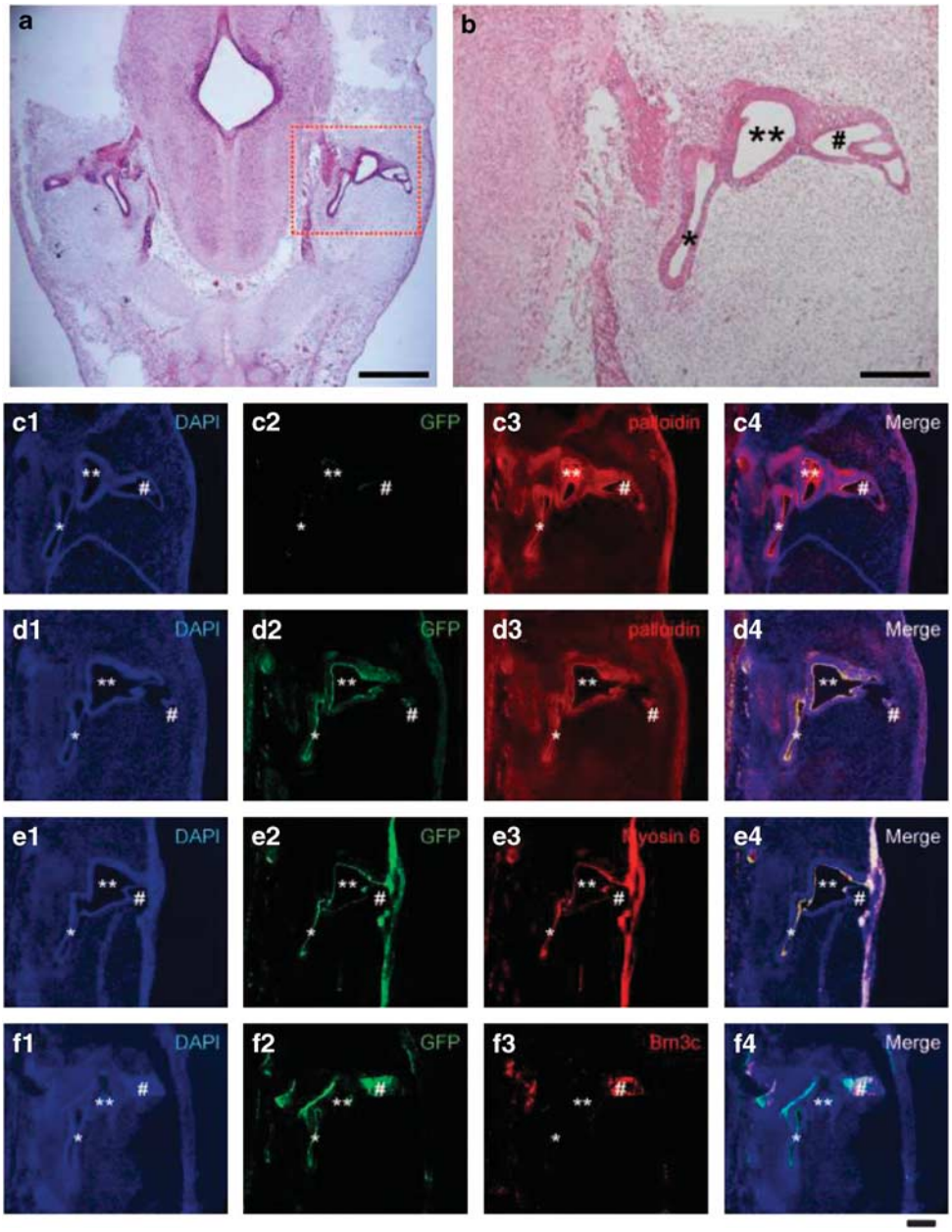

Figure 6 Transplantation of cells from EB outgrowths containing HC-like cells into chick embryos. (**a**) Graft cells were transplanted into the otic vesicle of chicken embryos. Three days after injection, normal morphological processes were confirmed by HE staining. Scale bar = 1 mm. (**b**) Enlarged image of area in (**a**) marked by dotted line. Scale bar = 150 μm. (**c**) No GFP-immuno-positive cells (**c**-2, green) were found in sections of the inner ear transplanted with undifferentiated ES cells. High phalloidin-positivity was observed in the inner ear (**c**-3, red). (**d–f**) GFP-immuno-positive cells (**d**-2, **e**-2, **f**-2, green) were found to be integrated in the developing inner ear transplanted with cells from EB outgrowths cultured with ST2-CM. Phalloidin-stained cells and GFP-immuno-positive cells were simultaneously observed in the inner ear (**d**-3, red). (**e** and **f**) Most of the integrated cells were immuno-positive for myosin6 (**e**-3, red) and Brn3c (**f**-3, red). Scale bar = 100 μm. Abbreviations: *cochlear duct; **lateral ampulla; #semicircular canal

the otic vesicles of chicken embryos.[21] Three days after injection, we performed morphological analyses (Figures 6a–f). Using H&E staining, normal morphological processes of the cochlear duct, lateral ampulla, and semicircular canal were confirmed (Figures 6a and b). ES-derived cells, shown as GFP-immuno-positive cells, were not detected in the developing inner ear tissues transplanted with undifferentiated ES cells (Figure 6c), whereas GFP-immuno-positive cells were found to be integrated in those transplanted with cells from EB outgrowths grown in ST2-CM (Figures 6d–f, Supplementary Figure S5). Phalloidin-stained cells were GFP-immuno-positive (Figures 6d2 and d3), while most of the ES-derived cells were integrated in an orthotopic manner and expressed HC-related markers, such as myosin6 and Brn3c (Figures 6e and f).

Discussion

ES cells are clonal cells derived from the inner cell mass of developing blastocytes that have an ability to proliferate

extensively *in vitro* and generate any cell type of the three embryonic germ layers.[15] Although the differentiation of ES cells into various cell types including neurons has been investigated in a number of studies, only a few reports have documented the differentiation of ES cells into HC-like cells.[18–21] Unfortunately, the methods used for such analysis are time consuming and complicated with multi-step processes, such as formation of EBs, expansion of nestin-positive cells, and exposure to several cytokines, and only result in a very small number of cells expressing HC-related markers. In the present study, we developed a single-medium culture method using CM from cultures of ST2 stromal cells (ST2-CM), by which EBs differentiated efficiently into HC-like cells. We termed this technique for inner ear hair cell induction by ST2-CM as the HIST2 method.

Stromal cell lines such as PA6 and MS5 have been employed for generating neurons from ES cells.[17,28] PA6 cells are known to generate mesencephalic dopamine neurons from ES cells with the SDIA method, which is considered to require cell-to-cell contact for efficient neural induction. In the present study, ES cells cultured with PA6-CM for 18 days expressed MAP2 and GFAP (Figure 1c), while they failed to express the HC markers Brn3c, myosin6, and α9AchR (Figure 2a). On the other hand, we found that ST2-CM induced EBs to express all of those HC markers. ST2 cells come from a stromal cell line established from mouse bone marrow.[29] They are known to have potential for forming eye-like structures from ES cells *in vitro* [30] and have been used to induce melanocytes from stem cells including ES cells *in vitro*.[31] However, there is no report regarding the use of ST2 cells for induction of HC-like cells from ES cells.

In the present study, we developed a simple single-medium culture method for differentiation of ES cells into HC-like cells. We screened several types of culture media including stromal cell conditioned media and found that CM from ST2 cells was able to efficiently induce HC-like cells. After the end of culturing with ST2-CM, more than 20% of the cells in EB outgrowths expressed HC-related markers, including Brn3c, myosin7a, calretinin, Math1, myosin6, and α9AchR, along with the formation of stereocilia-like structures. We believe that this HIST2 method is quite simple and highly efficient to obtain ES-derived HC-like cells in a relatively short cultivation time.

In an examination of the time-course of gene expression in 18-day cultures of 4-day EBs in regard to neural differentiation and HC development, nestin and myosin7a expressions were the first to appear by day 7, followed by myosin 6 and Math1 by day 11, and Brn3c and α9AchR by day 18 (Figure 5a), which seemed to mirror the developmental processes of HCs in the cochlea.[32] Furthermore, co-existence of $p27^{Kip1}$-immuno-positive SC-like cells in the culture together with calretinin-immuno-positive HC-like cells also suggests that EB outgrowths grown in ST2-CM provided a microenvironment resembling that in the developing inner ear. Interestingly, in addition to such cells with single immune positivity for $p27^{Kip1}$ or calretinin, cells expressing both $p27^{Kip1}$ and calretinin were found in the EB cultures with ST2-CM (Figure 5c). We considered that these double-positive cells were likely common progenitors for SC- and HC-like cells. However, it was recently reported that postnatal mouse cochlea-derived SCs have an ability to *trans* differentiate into HCs in culture.[27] Considering the possible conversion of SC- to HC-like cells, some of the double-positive cells may have been the result of retrograde differentiation of SC-like cells. Though further studies are necessary to analyze the generation of double-positive cells and their characteristics, it is noteworthy that SC- and HC-like cells, as well as cells possessing both of those markers were concomitantly generated from undifferentiated ES cells in the same culture.

In the experiment of transplantation using chick embryos, we observed integration of GFP-positive cells only when cells from ST2-CM-treated EB outgrowths were grafted, not those from undifferentiated ES cell colonies (Figures 6c–f). Although the reason why the undifferentiated ES cells failed to be integrated is unclear, the expression of adherent proteins such as integrins and cadherins might be an important factor.[33,34] In addition, integrated cells were located in the developing inner ear and expressed HC-related markers such as myosin6 and Brn3c, indicating that the cells had become integrated in the correct location and were functioning as host cells.

Despite the distinct induction of HC-like cells, the mechanisms by which the present HIST2 method affected the differentiation of ES cells are unknown. However, it was clearly shown that humoral factors without feeder cells, though not chemically determined, were sufficient for a high output of HC-like cells, because ST2-CM did not contain any cell components. The importance of humoral factors was also confirmed by ST2-CM dose-dependent gene expressions of myosin6, Math1, Brn3c, and α9AchR (Figure 2b). BMP family members are known to have a pivotal role in inner ear development[35] and ST2 cells were reported to express BMPs.[36] We confirmed the expression of BMP2 and BMP4 using RT-PCR method (Supplementary Figure S2). Therefore, we speculate that BMPs are important factors secreted from ST2 cells.

Previous reports have noted that ST2 cells promoted the differentiation of melanocytes from ES cells.[31] In the inner ear, melanocytes reside in the stria vascularis, and are required for normal development of the cochlea and for developed cochleae to function normally.[37] Although melanin pigment-bearing cells were not observed microscopically in EB outgrowths in ST2-CM, cells expressing c-kit were detected with immunocytochemical and RT-PCR methods (Supplementary Figure S3). Considering the existence of SC-like cells and possible melanocytes in addition to HC-like cells in EB outgrowths in ST2-CM, we think that the present HIST2 method provides a microenvironment that is suitable for not only HCs, but also various other types of cells residing in the cochlea.

In regard to CM-induced differentiation of ES cells, we speculate that ST2-CM might induce differentiation of ES cells into a wider range of cells than PA6-CM, including cell populations that assist with differentiation of nestin-positive HC-progenitor cells into mature HC-like cells. In the present experiments, we found that EBs expressed nestin and ζ-globin in ST2-CM, in contrast to only nestin in cultures with PA6-CM (Supplementary Figure S4).

In conclusion, we investigated the differentiation of ES cells into inner ear HCs using the differentiation inducing activity of

culture supernatants from stromal cells. CM from ST2 cells notably promoted the differentiation of ES cells into HC-like cells. The present method is simple and efficient to obtain HC-like cells with a relatively short cultivation period, and useful for elucidation of the developmental mechanisms of inner ear HCs.

Materials and Methods

Cells. Undifferentiated ES cells (EB3 and G4-2, a kind gift from Dr. Hitoshi Niwa, RIKEN Center for Developmental Biology, Kobe, Japan) were maintained in gelatin-coated dishes without feeder cells in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum ((FBS) Gibco, Invitrogen, Carlsbad, CA, USA), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 4.5 mg/ml D-glucose, and 1000 U/ml of LIF (Chemicon, Millipore, Billerica, MA, USA). EB3 and G4-2 cells were sublines derived from E14tg2a ES cells, and carried the blasticidin S-resistant selection marker gene driven by the Oct-3/4 promoter (active in undifferentiated state).[16] G4-2 ES cells were derived from EB3 ES cells and carried the *EGFP* (enhanced green fluorescent protein) gene under control of the CAG expression unit.

Stromal cell lines, ST2 cells and PA6 cells, were obtained from BRC (RIKEN Cell Bank, BioResource Center, Tsukuba, Japan) and maintained in DMEM supplemented with 10% FBS (Sigma).

Single-medium culture of EBs. We consider the present culture method to be simple to perform. EBs were cultured in single medium for 14 days (Figure 1a). To form EBs, ES cells dissociated from undifferentiated ES colonies by trypsin were cultured in hanging drops.[38] The cell density of each drop was 500 cells per 20 μl of ES cell medium in the absence of LIF (ES-M). After 4 days, the resulting EBs in hanging drops were collected and plated in plastic 100-mm gelatin-coated dishes (20 EBs per dish) in various media (Table 1), then allowed to attach to form outgrowths. Half of the culture medium was changed with new medium every 2 days during the 2-week EB outgrowth cultures.

CM was collected from PA6 or ST2 cells cultured in ES-M. Briefly, confluent 100-mm plates were washed with PBS and the cells were given new ES-M. After 48 h, the supernatant was collected, centrifuged for 5 min, filtrated with a 0.22-μm syringe membrane filter (Millipore, Billerica, MA, USA), and used as CM (ST2-CM, PA6-CM).

RT-PCR. Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA, USA) following the protocol of the manufacturer. Extracted RNA was then treated with DNase I (Takara, Otsu, Japan) to remove DNA. After inactivation of DNase I, 1 μg of total RNA was synthesized with first-strand cDNA with random primers and reverse transcriptase (M-MLV RT, Promega, Madison, WI, USA) for 1 h at 37 °C. For PCR analysis, 0.5 μg of cDNA was used as a template and amplified using the primer sequence shown in Supplementary Table S1. General PCR conditions were 25–30 cycles at 94 °C for 2 min, 94 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 1 minute. The PCR products were run on 1.5% agarose gels.

Immunocytochemistry and immunohistochemistry. Immunofluorescence analysis was carried out using standard protocols. Briefly, cells were fixed in 4% paraformaldehyde, then cellular membranes were permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA (T-PBS). Primary antibodies and dilutions in T-PBS were as follows: anti-GFP (1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Brn3c (1 : 400, Covance, Emeryville, CA, USA), anti-MAP2 (1 : 100, Santa Cruz), anti-calretinin (1 : 1000, Chemicon, Millipore), anti-Math1 (1 : 200, Chemicon, Millipore), anti-myosin6 (1 : 100, Santa Cruz Biotechnology), anti-α9AchR (1 : 100, Santa Cruz Biotechnology), anti-Espin (1 : 100, Santa Cruz Biotechnology), anti-$p27^{Kip1}$ (1 : 200, Biosource, Invitrogen, Carlsbad, CA, USA), anti-c-kit (1 : 100, Abcam, Cambridge, MA, USA), anti-acetylated tubulin (1 : 500, Abcam), and anti-myosin7a (1 : 500, kindly provided by Dr. A El-Amraoui, Institut Pasteur, Paris, France).[39] After incubation overnight at 4 °C and washing with TPBS three times, AlexaFluor 488 or 546 conjugated anti-goat, anti-rabbit, or anti-mouse secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used to detect primary antibodies. All nuclei were stained with DAPI (Dojin). After incubation for 1 h at room temperature and washing with TPBS three times, fluorescence was detected with a laser scanning confocal imaging system (Olympus, Tokyo, Japan).

Phalloidin staining. Cells were fixed in 4% paraformaldehyde, then cellular membranes were permeabilized with 0.3% Triton X-100 in PBS. After incubation for 10 min, RITC labeled-phalloidin (diluted 1 : 100 in PBS, Sigma) was reacted for 30 min and washed with PBS three times. All nuclei were stained with DAPI. Fluorescence was detected with a confocal microscope.

Scanning electron microscopy (SEM). Undifferentiated ES cells or cells cultured with ST2-CM for 18 days were re-plated on membrane filters (BD Bioscience, Bedford, MA, USA). After overnight cultivation, the membranes were washed with PBS and replaced with 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 45 min. The buffer was removed and the membranes were post-fixed with 1% osmium tetroxide in buffer (60 min), washed three times with fresh buffer (10 min each) and then distilled water (10 min), then dehydrated with an ethanol series and HMDS (Polysciences, Warrington, PA, USA) for 5 min. Finally, they were coated with osmium and examined using a SEM (S-5200, HITACHI, Hitachi City, Japan).

The organ of Corti was isolated from the inner ear of postnatal mice (day 1) and subjected to SEM observations.

Dye permeation experiment. To investigate the possibility that ES-derived HC-like cells contain functional mechanotransduction channels, FM1-43FX dye (Invitrogen) was used.[26] Cells were exposed to FM1-43FX (5 μM) for 10 s at room temperature, washed thoroughly in PBS, then fixed in 2% PFA and counterstained with DAPI. Furthermore, immunostaining for Brn3c was performed following FM1-43FX dye treatment and fixation. Cells were examined using confocal microscopy. Fields were chosen at random ($n = 15$).

Transplantation into chick embryos. Undifferentiated ES cells or cells from 14-day EB outgrowths in ST2-CM were dissociated with trypsin and injected using micro-glass pipettes into the right otic vesicles of day three chicken embryos (Hamburger and Hamilton stages 19–20). The left otic vesicles did not receive cell grafting and served as controls for immunohistochemistry. Specimens were fixed for 12 h in 10% formalin buffered-phosphate, dehydrated by an ethanol series, and embedded in paraffin. Cross-sections (6 μm) were cut using a microtome. Sections of the inner ear were prepared by axial cutting on day 6.5 of embryonic development. Immuno-labeling was initiated by de-paraffinization and blocking the sections for 1 h with TPBS, followed by the protocol described below. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Nara Medical University.

Statistical analysis. The fraction of immuno-positive cells among total cells was determined in a double-blind fashion by counting $\sim$300 cells in 15 randomly selected microscopic fields per experiment. Three experiments were conducted for each determination and significance was calculated using Student's *t*-test.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)