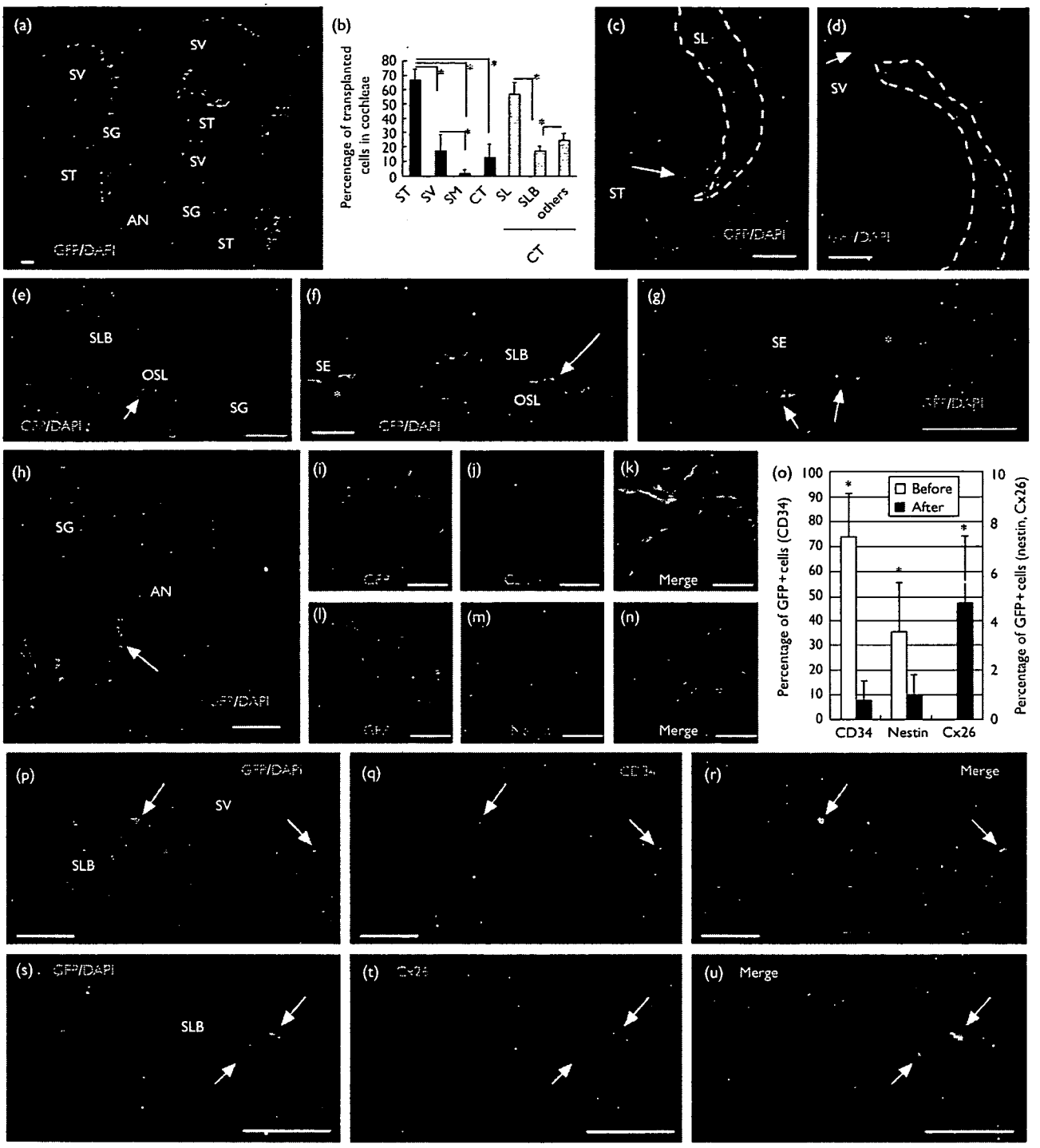

Fig. 1 Distribution of engrafted bone-marrow stromal cells in the cochlea and the expression of cell markers before and after transplantation. Transplanted cells expressing green fluorescent protein (GFP) were observed from the base to the apex of the cochleae, and were mainly located in the scala tympani (ST) and the scala vestibuli (SV) (a). Among the cochlear compartments, the transplanted cells were most frequently found in the ST (analysis of variance with the Scheffe's test, $^*P < 0.05$ in b) and in cochlear tissue (CT), the spiral ligament (SL) is the site in which transplanted cells were most frequently observed (b). Some of the transplanted cells in the ST (arrow in c) or the SV (arrow in d) were located adjacent to the SL (indicated by the dotted lines in c, d) in which transplanted cells were found. Transplanted cells were also observed in the spiral limb (SLB) (e, p, s), the osseous spiral lamina (OSL) (arrow in e, f), the sensory epithelium (SE) (asterisk in f, g), the connective tissue beneath the SE (arrows in g) and the acoustic nerve (AN) (arrow in h). Before transplantation, the BMSCs expressed CD34 (i–k) and a few were positive for nestin expression (l–n). The ratio of CD34 or nestin expression was significantly reduced after transplantation (unpaired t-test, $^*P < 0.05$ in o), and that of connexin26 (Cx26) expression was significantly increased after transplantation (o, the y-axis on the right side shows percentage for the CD34 ratio and the left for the ratio of nestin or Cx26). The transplanted cells that settled in the SV exhibited the expression of CD34 (arrows in p–r), and those in the SLB showed Cx26 expression (arrow in s–u). The scale bars represent 50 μm. Bars in b and o show standard deviations.

of BMSCs after transplantation. It is unclear whether BMSC-derived cells in the cochlea preserve the capability for secretion of growth factors displayed by BMSCs before transplantation [8]. Therefore, the potential of BMSC-derived cells in the cochlea for the secretion of growth factors should be examined to determine the ability of application of growth factors in the cochlea by BMSC transplantation.

Previously, we have demonstrated the delivery of a secreting protein to the inner ear by transplanting genetically manipulated fibroblasts without using virus vectors [7]. The fibroblasts were, however, distributed throughout the perilymphatic space of the cochlea and not within the cochlear tissues. These findings demonstrate the settlement of BMSC-derived cells within the cochlear tissues, particularly within the SL and SLB, after transplantation into the perilymphatic space of cochleae, suggesting the potential of BMSCs for migration into the SL and the SLB. These observed trends in the sites for BMSC migration indicate that the cells are suitable candidates for delivering genes in these regions, because delivery of cochlear constructive proteins may be required for the settlement of genetically manipulated cells in the regions in which encoded proteins should be expressed.

The gap junction network in the SL and the SLB has been suggested to play a crucial role in the maintenance of the endocochlear potential, which is necessary for hearing [13,14]. Our data demonstrate that some of the BMSC-derived cells that settled in the cochleae expressed Cx26, indicating the possible use of BMSC transplantation for restoration of the gap junction network in the cochlear connective tissues. The number of BMSC-derived cells expressing Cx26, however, may not be sufficient for the restoration of the gap-junction network. We should develop further strategies for increasing the number of BMSC-derived cells that settle within these regions to realize cochlear functional recovery by BMSC transplantation.

Immunohistochemical analyses in this study demonstrated no transdifferentiation of BMSCs into the neural or epithelial lineage after transplantation into the cochleae. In contrast to these findings, previous studies have demonstrated that BMSCs can differentiate into various types of cells including a neural lineage [15,16]. Naito *et al.* [17] have reported a differentiation of BMSCs into neurons after transplantation into the modiolus of chinchilla cochleae that had been damaged by aminoglycosides; however, the number of BMSC-derived cells expressing a neural marker is very limited. In this study, we identified the expression of nestin in BMSCs before and after transplantation, although the number is very limited. We therefore consider that neural induction of BMSCs and selection of neural progenitors from BMSC-derived cells before transplantation might be necessary to achieve the restoration of cochlear neurons through the transplantation of BMSCs.

Conclusion

In summary, our current findings demonstrate that BMSCs have the capability to survive in the cochlea and migrate into the cochlear tissues, which indicates possible use of BMSC transplantation as a strategy for the treatment of SNHL. Further studies are, however, necessary to realize the practical use of BMSC transplantation for the treatment of inner ears.

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Effects of bone morphogenetic protein 4 on differentiation of embryonic stem cells into myosin VIIa-positive cells

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Abstract

Conclusion: Our results indicate that myosin VIIa-positive cells are generated from embryonic stem cells (ESCs) co-cultured with PA6 cells; however, bone morphogenetic protein 4 (BMP4) may not be a key molecule for induction of myosin VIIa-positive cells from the ESCs. *Background:* ESCs have been considered as a basis for cell therapy in a range of organs, because of their potential for self-renewal and pluripotency. Co-culture with PA6 stromal cells can induce differentiation of ESCs into various types of ectodermal cells including sensory progenitors. BMP4 plays an essential role in the development of sensory hair cells in the inner ear. *Materials and methods:* We examined effects of BMP4 on differentiation of ESCs into the hair cell immunophenotype. BMP4 was supplemented at different time points to ESCs co-cultured on PA6 stromal cells. The ESCs were then collected and examined for the expression of myosin VIIa, a hair cell marker, and βIII-tubulin, a neural marker. The expression of myosin VIIa and βIII-tubulin was identified. *Results:* Quantitative assessments revealed that exogenous BMP4 has significant effects on the expression of βIII-tubulin, but not of myosin VIIa.

Introduction

Bone morphogenesis protein 4 (BMP4) plays essential roles in the development of the inner ear including sensory epithelium [1–3]. Recently, it has been reported that BMP regulates differentiation and cell proliferation in developing auditory epithelia of chicks [4]. Hair cell regeneration has been a central issue in the field of inner ear research for decades. However, hair cell regeneration in mammalian cochleae is a problem that is still being discussed. Recent studies using gene transfer by adenovirus vectors have demonstrated the potential of supporting cells for transdifferentiation into hair cells [5,6]. However, in severely damaged cochleae, there are no remaining supporting cells that are capable of transdifferentiation into hair cells. In such circumstances, cell transplantation may be a possible strategy for hair cell regeneration.

Embryonic stem cells (ESCs) have been considered as a basis for cell therapy in a range of organs, because of their capability for self-renewal and pluripotency. Recently, it has been reported that ESCs can differentiate into inner ear cells including sensory hair cells [7]. We have also examined the potential of ESCs as a source of transplants for regeneration of spiral ganglion neurons [8,9]. ESC-derived cells following induction of differentiation into ectodermal cells are desirable, because most inner ear cells are originated from the ectoderm. Several methods for ectodermal induction of ESCs have been established. We have used the stromal cell-inducing activity (SDIA), in which ESCs are co-cultured with PA6 cells [10], stromal cells derived from mouse skull bones, for this purpose [8,9]. SDIA treatment can induce various types of neuronal cells with a combination of supplements of BMP4 or sonic hedgehog [11]. However, the potential of SDIA treatment for induction of inner ear sensory cells has not been examined. The aim of this study was set to determine the effects of BMP4 on differentiation of SDIA-treated ESCs into inner ear sensory cells. The profile of differentiation of SDIA-

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treated ESCs by exposure to BMP4 at various time points was analyzed by immunocytochemistry.

Materials and methods

Materials

Mouse G4-2 ESCs (donated by Hitoshi Niwa of Riken CDB, Kobe, Japan) derived from the E14tg2a ESC line [12], and carrying the enhanced green fluorescence protein (EGFP) gene driven by the CAG promoter, were used in this study. The PA6 cells (RCB1127), a stromal cell line derived from newborn mouse calvaria were provided from Riken Cell Bank, Kobe, Japan. Glasgow's Modified Eagle's Medium (GMEM), knockout serum replacement (KSR), and nonessential amino acids (NEAA) were purchased from Invitrogen (Carlsbad, CA, USA), pyruvate was from Sigma (St Louis, MO, USA) and 2-mercaptoethanol (2-ME) was from Wako, Osaka, Japan. Recombinant human BMP4 was from R&D Systems (Cleveland, OH, USA).

Induction of differentiation of ESCs

We used the SDIA for neural induction of ESCs [10,11]. ESCs were cultured to form differentiated colonies on a feeder layer of PA6 stromal cells derived from newborn mouse calvaria in GMEM supplemented with 5% KSR, 1 mM pyruvate, 0.1 mM NEAA, and 0.2 mM 2-ME at 37°C in a 5% CO_2 atmosphere. We set six conditions for the culture of ESCs during SDIA treatment according to the duration of the exposure to BMP4 at a concentration of 0.5 nM; during day 1–6, 2–6, 3–6, 4–6, 5–6 or no exposure. Colonies that formed on the PA6 monolayer after 6 days of culture were isolated by collagenase B (Roche Diagnostics, Tokyo, Japan). Cell suspensions of SDIA-treated ESCs were adjusted to a concentration of 10^3 cells/μl in GMEM, and a 10 μl portion of the cell suspension was then replaced onto a sterile membrane (Falcon™ Cell Culture Insert; 3.0 μm pore size, 24-well format; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in a 24-well culture plate (Asahi Techno Glass Corp., Tokyo, Japan). SDIA-treated ESCs were incubated in the culture medium containing no BMP4 for an additional 7 days. Each experimental condition consisted of five wells.

Immunocytochemistry

Following incubation, the cultured specimens were washed with phosphate-buffered saline (PBS; Nacalai Tesque Inc., Kyoto, Japan) and fixed with 4% paraformaldehyde in PBS for 15 min. The fates of cultured ESCs were determined by immunostaining for myosin VIIa, a marker for hair cells, Pax-2, a marker for sensory progenitors in the inner ear [13], and βIII-tubulin in whole mounts. ESC-derived cells obtained from four wells in each culture condition were double-stained with rabbit anti-myosin VI (×700; purchased from Tama Hasson, University of California, San Diego, CA, USA), and mouse anti-βIII-tubulin (×200; Covance, Berkeley, CA, USA). Secondary antibodies used were Alexa-Fluor 546-conjugated anti-rabbit and Alexa-Fluor 633-conjugated anti-mouse goat antibodies (×200; Molecular Probes, Eugene, OR, USA). Immunocytochemistry for rabbit anti-Pax-2 (×200; Covance) was performed in one well for each condition. The specimens were viewed with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany). In each well, we counted the numbers of ESC-derived colonies labeled by EGFP and those of colonies containing myosin VIIa- or βIII-tubulin-positive cells. The ratio for each marker-expressing colony was then calculated. The differences in the ratio for each marker among culture conditions were statistically analyzed by ANOVA with Fisher's protected least significant difference (PLSD). A p value <0.05 was considered significant.

Results

Both βIII-tubulin- and myosin VIIa-positive colonies were found in all the culture conditions. In these colonies, the majority of myosin VIIa-positive cells were located in the central portion of the colonies surrounded by βIII-tubulin-positive cells (Figure 1A–C). A few myosin VIIa-positive cells were found in the peripheral lesion of the colony. Immunostaining for βIII-tubulin demonstrated massive elongation of neurites from ESC-derived cells (Figure 1D–F). On the other hand, we found no cells that exhibited the expression of both myosin VIIa and βIII-tubulin. We found one or two ESC-derived colonies containing Pax-2-positive cells in cultures that were exposed to BMP4 during day 2–6, day 5–6 or no exposure (Figure 2).

Quantitative assessment for the ratio of βIII-tubulin-positive colonies demonstrated significant effects of BMP4 application on differentiation of ESCs into neurons ($p=0.0002$; Figure 3A). The differences in the ratio of βIII-tubulin-positive colonies between day 1–6 or 2–6 and day 4–6, 5–6 or no exposure, and between day 3–6 and day 5–6 or no exposure were significant at Fisher's PLSD. Early exposure to BMP4 reduced the ratio for βIII-tubulin-positive colonies. On the other hand, BMP4 application had no significant effect on the ratio for myosin VIIa-positive colonies (Figure 3B).

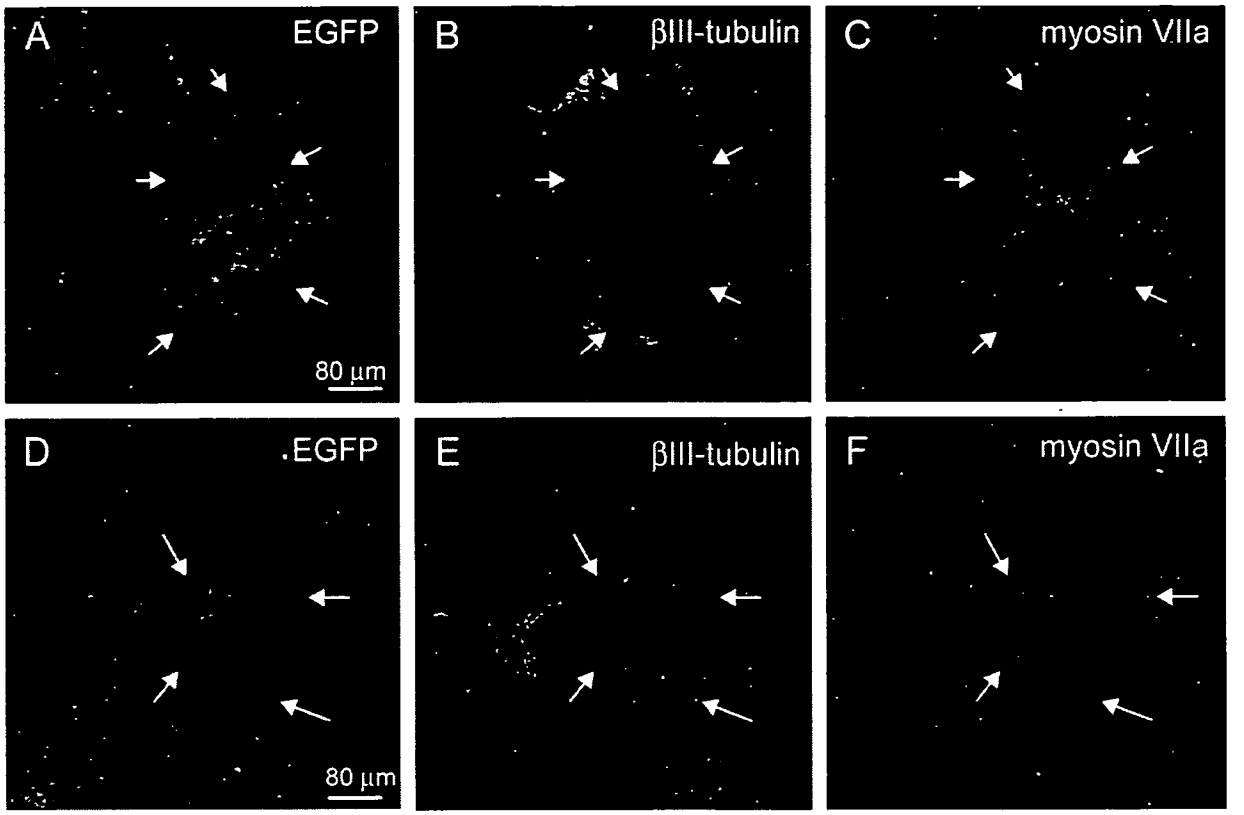

Figure 1. Expression of βIII-tubulin and myosin VIIa in SDIA-treated embryonic stem cells. (A–C) Embryonic stem cell colony exposed to BMP4 during day 3–6. (D–F) Embryonic stem cell colony cultured without exposure to BMP4. Arrows indicate the location of myosin VIIa-positive cells.

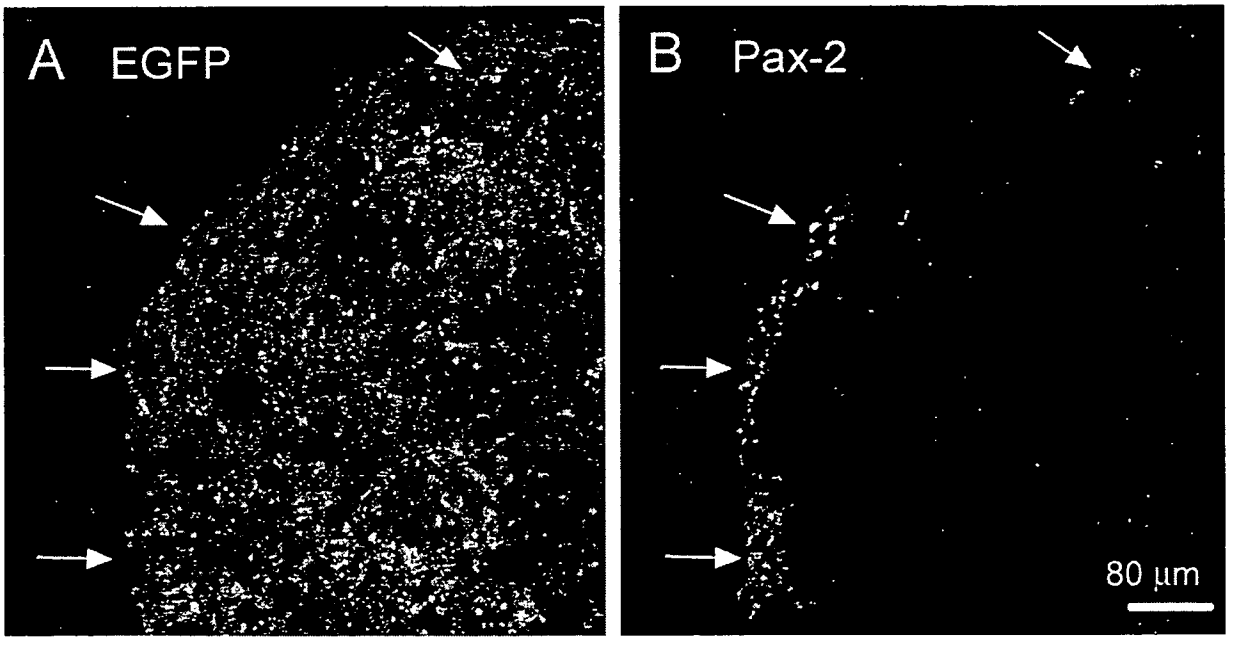

Figure 2. Expression of Pax-2 in SDIA-treated embryonic stem cells. Some embryonic stem cells exposed to BMP4 during day 2–6 exhibit the expression of Pax-2 (arrows).

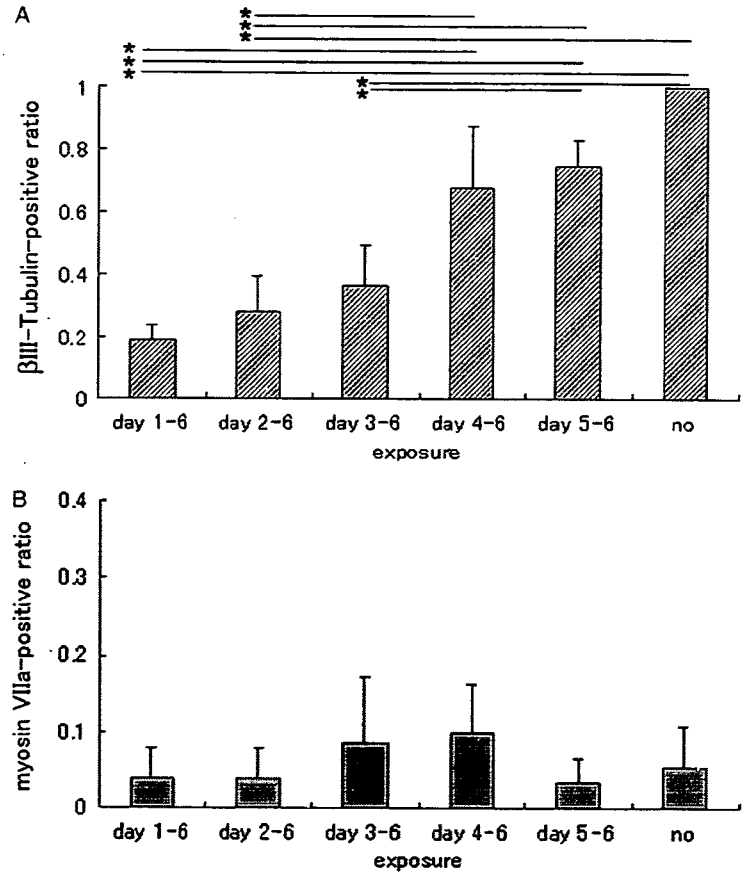

Figure 3. Quantitative assessment of the expression of βIII-tubulin and myosin VIIa in colonies of embryonic stem cells. X-axis shows the duration of exposure to BMP4. Exposure to BMP4 has significant effects on the expression of βIII-tubulin (A), and asterisks indicate significant differences in pairwise comparison. No significant differences were found in the ratio for myosin VIIa expression (B).

In culture conditions exposed to BMP4 from day 3 or 4 to day 6, the ratios for myosin VIIa expression were slightly higher than other culture conditions; however, no significant differences were found among experimental groups.

Discussion

Inner ear cell progenitors are derived from the ectoderm similar to neural or epidermal progenitors. The inner ear is derived from a thickened patch of ectodermal cells, which develops lateral to the developing hindbrain. The SDIA can generate various types of ectodermal cells from ESCs [11]. The present findings demonstrate that myosin VIIa- or Pax-2-positive cells can be derived from SDIA-treated ESCs, which indicates that hair cells may be generated from SDIA-treated ESCs.

Early exposure of SDIA-treated ESCs to BMP4 induces epidermal differentiation of ESCs, while no exposure to BMP4 during the SDIA treatment results in highly neural differentiation of ESCs [11]. In addition, sensory progenitors are generated from SDIA-treated ESCs by the late exposure to BMP4 [11]. We therefore expected that late exposure of SDIA-treated ESCs to BMP4 might have the activity for differentiation of SDIA-treated ESCs into inner ear cells including hair cells. The present findings demonstrate that BMP4 has a significant effect on suppression of neural differentiation of SDIA-treated ESCs, which is identical to previous findings [11], indicating that the exposure to BMP4 certainly works in our culture systems. However, our results indicate no significant effects of BMP4 exposure on the expression of myosin VIIa in SDIA-treated ESCs. In addition, Pax-2-positive cells were generated with or without exposure to BMP4. These findings indicate that BMP4 exposure has no effects on differentiation of SDIA-treated ESCs into inner ear hair cells.

Recent studies on the development of the inner ear have indicated that BMP4 plays a crucial role in maturation of inner ear sensory epithelia [1–3]. In the chick otocyst culture system, BMP4 induces differentiation of hair cells from their progenitors, and promotes down-regulation of Pax-2 protein in

sensory epithelial progenitors, leading to reduced progenitor cell population [4]. Based on these findings, the early exposure to BMP4 might induce reduction of hair cell progenitors derived from ESCs, and the late exposure to BMP4 might induce differentiation of hair cells from the progenitors derived from ESCs. However, our findings demonstrated no down-regulation of Pax-2 in ESC-derived cells by the early exposure to BMP4 and no up-regulation of myosin VIIa by the late exposure. Therefore, the molecules that determine the fate of ESCs to differentiate into inner ears in the earlier stage of development may be required for induction of differentiation of ESCs into inner ear cells.

In conclusion, myosin VIIa-positive cell populations were obtained from SDIA-treated ESCs; however, no significant effects of BMP4 exposure on the expression of myosin VIIa in ESC-derived cells were identified. Further studies are required for identification of key molecules for induction of differentiation of SDIA-treated ESCs into hair cells.

Acknowledgements

We thank H. Niwa for providing ESCs, T. Hasson for providing the antibody for myosin VIIa, S. Sasai for providing PA6 cells, and R. Sadato and Y. Nishikawa for technical assistance. This study was supported by a Grant-in-Aid for the Regenerative Medicine Realization project from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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Hair cell differentiation becomes tissue specific by E9.5 in mouse inner ear

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Tissue culture is a standard method to study tissue interactions during embryogenesis. The serum that is usually used in culture can, however, confound results as it includes unidentified factors. In this study, we used a serum-free otocyst culture to investigate the tissue interactions that determine hair cell fate in mice otocysts. Otocysts cultured with surrounding tissues have the ability to produce mature hair cells in serum-free otocyst culture. Although isolated otocysts from E9.5 mice produced hair cells, those from E9.0 mice could not. This indicates that the mouse otocyst gains the ability to generate hair cells between E9.0 and E9.5 and that this ability depends on signals from the surrounding mesenchyme and/or the hindbrain. *NeuroReport* 18:841–844 © 2007 Lippincott Williams & Wilkins.

Keywords: differentiation, inner ear, organ culture

Introduction

The vertebrate inner ear transduces the mechanical vibrations that result from sound into electrical impulses. This function is performed by specialized mechanoreceptors, the hair cells. The development of these cells results from a series of signals emanating from surrounding tissues. Initially, extrinsic signals specify the precursor of the inner ear, the otocyst to form; in the mouse this occurs at E8.5 [1]. Patches of sensory progenitors are then specified and these will give rise to the hair cells and associated supporting cells. Despite the wealth of information about the intrinsic factors that specify these processes (reviewed by Barald and Kelley [2]), information about the role of extrinsic factors in directing hair cells to form from the otocyst is limited. To elucidate the source of extrinsic signals acting on otic cells to promote hair cell development, we devised an *ex vivo* serum-free otocyst (SFO) culture. Tissues that affect hair cell development were identified using this culture method. We find that the periotic mesenchyme and the hindbrain have profound effects on the development of the hair cells.

Methods

Serum-free otocyst culture

Pregnant ICR mice (Japan SLC, Shizuoka, Japan) were dissected and each embryo was staged individually according to the Edinburgh Mouse Atlas (*http://genex.hgu.mrc.ac.uk/*). Embryos ranging from E8.5 to E10.5 were used for this experiment. All animal procedures complied with institutional and governmental guidelines and were approved by the Animal Care Committee of the RIKEN Kobe Institute, which are in concordance with the NIH Guide for the care and use of laboratory animals.

Otocysts were dissected with or without surrounding tissue using sharpened tungsten wires (0.3-mm diameter, no coating, MT Giken, Tokyo, Japan). Surrounding tissue included the adjacent hindbrain (rhombomeres 4 and 5) and periotic mesenchyme, although endoderm was excluded (OeNM, Fig. 1b). For cultures containing only otic epithelia (Oe, Fig. 1a), periotic mesenchyme was removed manually. Dissected tissues were placed in a collagen drop as described previously [1], then flooded with 200 µl of Dulbecco's modified Eagle's medium (DMEM, with 4500 mg glucose/L and 584 mg L-glutamine/L; Sigma, St. Louis, Missouri, USA) supplemented with 10% Knock-Out Serum Replacement (KSR; Invitrogen, Carlsbad, California, USA) and penicillin G (100 U/ml; Meiji Seika Kaisha, Tokyo, Japan). To ensure the best survival of SFO cultures, only half of the media was changed daily. After the required length of incubation, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 60 min at room temperature.

Immunohistochemistry

Samples were assayed using standard immunofluorescence methods in the whole mount collagen gel drop. The following primary antibodies were used: rabbit anti-myosin VIIa (provided by Tama Hasson, University of California, San Diego, California, or Proteus Bioscience, Ramona, California, USA, 25-6790; diluted 1:1000), rabbit anti-myosin VI (Tama Hasson; diluted 1:400), rat anti-E cadherin (ECCD-2, Takara Bio, Shiga, Japan, M108; diluted 1:500),

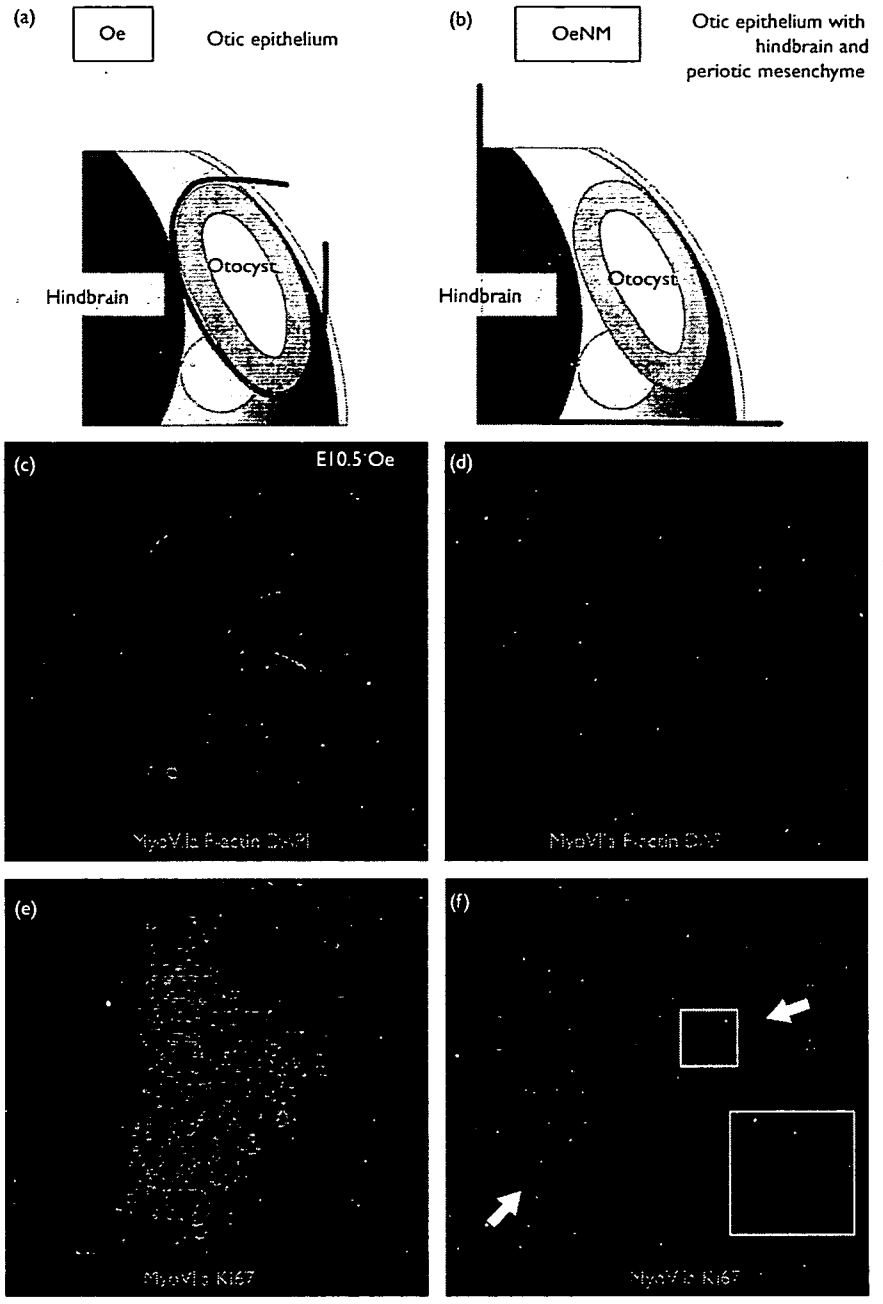

Fig. 1 Otic epithelium explants give rise to hair cells in serum-free otic (SFO) culture. Otocysts were isolated as otic epithelium only explant (a, Oe) or with adjacent hindbrain and periotic mesenchyme (b, OeNM). E10.5 mouse otocysts (Oe) were cultured in serum-free media for 7 (c, d, e) or 10 days (f), and assayed by immunohistochemistry. E10.5 otocysts in SFO cultures form a complex three-dimensional morphology (c). Higher magnification of a typical inner ear flask-shaped hair cell, positive for myosin VIIa (d). Clusters of hair cells show a configuration reminiscent of vestibular (e) and cochlear (f) hair cells. A pair of white arrows in panel (f) indicates a linear gap dividing a myosin VIIa (+) cluster. The inset shows the magnification of the small square in the picture, which shows two or three rows of inner hair cells and many rows of outer hair cells separated by a linear gap (pillar cells).

goat anti-parvalbumin (Swant, Bellinzona, Switzerland, PVG-214; diluted 1:5000), and mouse anti-Ki67 (Becton-Dickinson, Franklin Lakes, New Jersey, USA, 556003; diluted 1:200). Primary antibodies were detected by secondary antibodies conjugated to AlexaFluor 488, 546 or 647 (Invitrogen). For the detection of F-actin, AlexaFluor 488 conjugated phalloidin was used. Blocking solution consisted of 5% normal goat serum or 5% donkey serum in 0.05% Tween-20 in phosphate-buffered saline. Samples were counter-stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen). After the staining procedure, the collagen gel was removed by forceps and microscissors, mounted between two cover slips with SlowFade antifade reagent (Invitrogen) then imaged by using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Exton, Pennsylvania, USA). For semiquantification of the number of myosin VIIa positive cells in sensory patches of each explant, three

confocal images that included myosin VIIa positive clusters and each separated more than 20 μm were taken with a ×40 objective lens. Samples were categorized into three groups by the average number of myosin VIIa positive cells in those optical planes; 0 cells (no myosin VIIa positive cells), one to five cells (one to five myosin VIIa positive cells in one confocal image), and more than six cells (more than six myosin VIIa positive cells in one confocal image).

Results

Hair cell development in serum-free otocyst culture

Otocysts of E10.5 mouse embryos were explanted (Oe, Fig. 1a) and kept in SFO culture. After 7–10 days of culture, they possessed cells of a typical hair cell morphology (Fig. 1d), which is a flask-shaped cell with a basally deviated nucleus, narrowed at the apical end with cuticular plate-like actin accumulation on the apical side. These cells showed strong immunoreactivity for the hair cell markers, myosin VIIa (Fig. 1c–f), myosin VI and parvalbumin. When embryonic inner ears are cultured, vestibular hair cells usually appear as a simple cluster, and cochlear hair cell arrangement is disturbed; one to three rows of inner hair cells and many rows of outer hair cells are separated by a line of pillar cells. In SFO cultures, hair cells appeared in clusters resembling vestibular (Fig. 1e) or cochlear (Fig. 1f) hair cell patterns.

To determine the temporal profile of hair cell appearance, E10.0 otocysts without surrounding tissue (Oe) were cultured for 3–7 days *in vitro* (DIV). After 3 DIV only one of the 11 explants showed immunoreactivity against myosin VIIa; however, after 4 DIV, progressively more explants, with larger clusters of myosin VIIa positive cells, were observed until 6 DIV, where myosin VIIa was observed in a higher number of explants. Further longer culture periods did not increase the number and size of the clusters of myosin VIIa positive hair cells (Fig. 2).

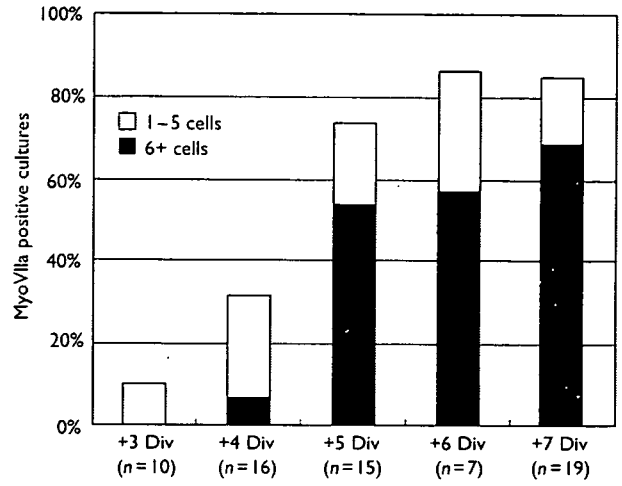

Fig. 2 Inner ear hair cells can be detected after 4 days *in vitro*. Graph showing the number of explants positive for the hair cell marker myosin VIIa. Oe explants from E10.5 embryos were cultured for 3–7 days. Hair cell development can be detected in 30% of cultures after 4 days *in vitro*. The number of positive cultures increases until 6 DIV. The number of explants with very small clusters of myosin VIIa positive cells (containing less than five cells) is shown as white boxes. Explants with larger clusters are shown as black boxes. Total numbers are shown below.

Table 1 Percentage of Oe and OeNM explants positive for hair cells

Explanted stage	Culture period (days)	Myosin VIIa positive cultures	
		Oe	OeNM
E9.0	7	1/11 (9.1%)	22/43 (51.2%)
E9.5	6.5	28/48 (58.3%)	22/43 (51.2%)
E10.0	6	33/38 (86.8%)	8/16 (50.0%)
E10.5	5.5	45/52 (86.5%)	23/26 (88.5%)

E9.5 otocysts can produce hair cells without any additional factor

Otocysts were explanted from E9, E9.5, E10, and E10.5 mouse embryos with or without surrounding tissue and cultured as above (Oe and OeNM, respectively). The duration of the cultures was adjusted such that upon fixation the explants would be equivalent to E19.5 or older, a time when all the cells fated to become hair cells show clear positivity for myosin VIIa. OeNM cultures taken from mice embryos of any stage formed hair cells possessing myosin VIIa staining and apical F-actin (in over 50% of cases). In contrast, hair cells only formed in Oe explants taken from E9.5 and older embryos; virtually no E9 Oe explants generate hair cells (Table 1). Further increase (more than 80%) in the positivity for myosin VIIa in E10.0 Oe, E10.5 Oe, and E10.5 OeNM explants was observed.

Discussion

Tissue culture has been used for studies identifying the tissue source of particular signals that can influence the development of the inner ear and as an assay for developmental factors [1]. By explanting the inner ear with different proportions of surrounding tissue, it is possible to make predictions about the influence that tissue has on inner ear development. As Van de Water and Ruben [3] first successfully cultured mammalian inner ear, serum containing media have been used. The use of serum, which does include undefined factors, may, however, lead to a false picture of inner ear development. In this study, we used KSR instead of serum, which is used as a standard supplement for serum-free embryonic stem cell culture. KSR does not contain FGFs, EGFs, TGFβ, TGFα, BMPs, retinoic acid, IGFs, estrogen, and Wnts (information from Invitrogen Technical Support). Thus, this culture system is suitable for a more defined culture environment.

In our SFO culture, mouse otocysts gave rise to hair cells that have a typical morphology and express multiple specific markers. The timing of hair cell appearance was also preserved; E10.0 otocysts showed no hair cells after 3 DIV, although these progressively increased and finally plateaued at 6 DIV. This correlates with published data and our unpublished observations showing myosin VIIa immunoreactivity in the otocyst at E13.5–14 (corresponding to 3.5–4 DIV of E10.0 SFO culture) [4,5]. As development progresses the number of myosin VIIa positive cells also increases. By E17.5 (equivalent to 7 DIV) the number of myosin VIIa positive hair cells remains constant. Thus, the dynamics of hair cell development are recapitulated in SFO cultures.

The autonomy of the otocysts that enables them to generate hair cells suggests the culmination of the extrinsic

specification events. Although isolated E9.5 otocysts (Oe) in SFO culture robustly generated hair cells, E9.0 Oe SFO cultures do not. The fact that E9.5 Oe does not require additional factors to produce hair cells apparently conflicts with recent data showing a requirement of cochlear hair cell differentiation for periotic mesenchyme, and more specifically EGF, from mouse embryos as late as E13.5 [6]. In that study dissociated cochlear cells were used and it is possible that the disruption of the otic epithelium (the experimental paradigm necessarily used in that study) causes a stress on the otic tissue that can only be rescued by EGF or mesenchymally derived EGF.

As reviewed by Romand *et al.* [7], commitment of cells in the otocyst to a specific cell fate occurs very early during embryogenesis and is controlled both by intrinsic and extrinsic factors like retinoic acid. Corresponding to this, our data indicate the existence of signals acting on the mouse otocysts between E9.0 and E9.5, which induce hair cells. Alternatively, it is possible that before E9.5 hair cell progenitors require a signal for their maintenance. These experiments cannot distinguish the two possibilities. Combining SFO culture with an analysis of the earliest markers of hair cell progenitors, however, while excluding the possibility of cell death using apoptosis markers, can more completely elucidate the function of the surrounding tissue.

We note that there is an apparent increase in the ability of SFO cultures to give rise to hair cells; E10 Oe will give rise to significantly more hair cells than E10 OeNM. This suggests the existence of another factor emanating from hindbrain or periotic mesenchyme at E10 that may delay or prevent precursor cells from adopting a hair cell fate. Further experiments are also underway to confirm this.

Conclusion

We developed a SFO culture using KSR to eliminate effects of unidentified factors in serum. Mice otocysts developed, largely recapitulating developmental dynamics *in vivo* in this culture system. Using SFO culture, we showed that E9.5 otocysts could give rise to hair cells without any additional factor, and this autonomy is provided by periotic mesenchyme or adjacent hindbrain (rhombomeres 4 and 5) between E9.0 and E9.5.

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