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Original Article

Effect of Growth Factor Supplementation on the Hair Cell Specific Markers of Cells Harvested from Basilar Membrane

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OBJECTIVE: Loss of auditory hair cells is a major cause of deafness. The presence of auditory progenitor cells in the inner ear raises the hope for mammalian inner ear cell regeneration. In this study, we aimed to investigate the effect of growth factor supplementations, namely a combination of epidermal growth factor (EGF), insulin-like growth factor (IGF), and beta (β)-fibroblast growth factor (βFGF), on the expression of hair cell-specific markers by cells harvested from the cochlear membrane. This would provide an insight into the capability of these cells to differentiate into hair cells.

MATERIALS and METHODS: EGF, IGF, and βFGF were supplemented into the culture medium. The cells were evaluated by morphology, growth kinetic, gene expression, and protein expression.

RESULTS: The cultured cells of mouse basilar membrane were spindle shaped. Growth factors-enriched medium promotes a significantly higher proliferative activity than the basic culture medium but did not alter the cell morphology. Growth factors-enriched medium did not show any significant differences in the protein expression of the hair cell-specific markers myosin VIIa and calretinin and the stem-cell marker nestin. Gene expression analysis showed that the expression of the hair cell-specific genes myosin VIIa and calretinin as well as the stem cell genes nestin, Rex1, and Sox2 was reduced after the cells were passaged in the growth factor-supplemented medium. Cells in the basic medium expressed a significantly higher level of hair cell-specific genes at certain passages.

CONCLUSION: Growth factor supplementation could not maintain the expression of hair cell-specific markers by cells obtained from the cochlear membrane.

KEYWORDS: Auditory progenitor cells, hair cell regeneration, in situ culture digestion, hair cells, stem-cell markers, regenerative medicine

INTRODUCTION

Inner ear consists of cochlea and vestibular organs, which are the receptors for hearing and head movement [1]. The inner ear contains cells with mechanoreceptors, which are known as hair cells, and they are surrounded by supporting cells. The degeneration and death of hair cells is a major cause of hearing disorders, which can be caused by aging, excessive noise, infections, and drugs [2]. The number of hair cells is very small, and their loss is associated with compromised hearing disability [3]. Although hair cells can spontaneously regenerate in birds and lower vertebrates, the mature mammalian cochlea does not have this capability [4].

Auditory progenitor cells have been isolated and cultured from the early, postnatal mouse cochlear tissue; they contain properties of stem cells, which are involved in development of and differentiation to adult hair cells [5]. Myosin VIIa, calretinin, and espin are important in the development and function of hair cells, and they have been used as hair cell markers [6, 7]. Studies on the development and regeneration of hair cells by the differentiation of stem cells using various chemical and genetic modifications have provided hope for hair cell regeneration in humans [8]. The molecular factors responsible for promoting self-renewal and formation of adult hair cells have been actively sought [9, 10]. As adult hair cells cannot regenerate, auditory progenitor cells become the best source for hair cell regeneration. It has been proven that post-mitotic cells purified from the postnatal mouse cochlea retain the ability to divide and differentiate into new hair cells in culture [11]. These cells are sensitive, and it is important to obtain a favorable long-term culture condition that can result in highest yield without changing the phenotype. Several growth factors have been used, name-

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ly insulin-like growth factor (IGF), beta (β)-fibroblast growth factor (βFGF), and epidermal growth factor (EGF) [12, 13]. These studies have shown the positive effect of the growth factors on short-term culture. It is important to describe the effect of these factors on long-term and multiple-passage cell culture. Thus, in this study, we evaluated the ability of cells harvested from basilar membrane to sustain the long-term stability of auditory progenitor cells so that cell populations can be maintained and expanded with the aid of IGF, βFGF, and EGF. The in situ culture digestion method was used to obtain cells from the basilar membrane [14, 15].

MATERIALS and METHODS

Cell Isolation and Culture

This study was approved by the Institutional Research and Ethical Committee. The study was conducted at the Tissue Engineering Centre, University Kebangsaan Malaysia Medical Centre; a total of 18 mice were used in this study. The whole study was completed in a 1-year period. Cells were harvested from the basilar membrane from an average of 2-week-old mice. Harvest of the cochlear tissue started with a transcanal approach; the cartilaginous part of the external auditory canal was truncated, allowing access to the tympanic membrane. A tympanomeatal flap was raised using a sickle knife, which allows direct access to the tympanic bulla. Then, the bony bulla was picked open with a right-angled hook, and this exposes the bony labyrinth and promontory. At this stage, the round window membrane was identified with the stapedial artery situated caudally to it. A curved needle was used to free the edges of the bony labyrinth; fine scissors were then used to cut the bone that was cranial, caudal, and dorsal to the labyrinth; the temporal bone was completely removed. Soft tissues were subsequently dissected away from the labyrinth. A curved needle was placed within the round window membrane and used as a pick to gently uncap the cochlea, thereby exposing its contents. The membranous part of the labyrinth was then picked up from the bony labyrinth and placed directly into the culture medium. The reagents used in this study were purchased from Gibco, Invitrogen Corporation, California, USA, unless otherwise stated. Basilar membrane was washed with phosphate buffered saline (PBS) and digested with a 0.3% of collagenase Type I for 2 h at 37°C without agitating the sample. Cells isolated from a total of two cochleae were placed in the well of a 12-well plate. The cells were incubated at 37°C with 5% CO^2 either in basic growth medium (BGM) [F12/Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 1% Glutamax™, 1% Vitamin C (Sigma, Dorset, UK), and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)] or in growth factor-enriched medium (GFEM) [BGM supplemented with IGF (50 ng/mL), BFGF (20 ng/mL), EGF (50 ng/mL) (Peprotech Inc.; New Jersey, USA), and N-2® and B-27® supplements]. Media were replaced every 3 days. In average, cultured cells become confluent within 2 weeks, and this primary culture (P0) was trypsinized with 0.05% trypsin-EDTA and passaged to P1. Isolated cells harvested from the basilar membrane (auditory cells) were expanded until the passage P3 (n=6).

Growth Profile Analysis

The morphological features of cultured cells were examined everyday using inverted light microscope (Olympus, Shinjuku-ku, and Tokyo) and recorded. At each passage, cells were trypsinized as before on reaching a confluency of 80%. Total cell yield and viability at each passage was determined by the trypan blue dye exclusion assay. The growth rate (cells/day/cm^2) of cells was then calculated and documented at every passage.

Gene Expression Analysis

Total RNA of the cultured cells at each passage (P0–P3) was extracted using TRI reagent (Molecular Research Center, Ohio, USA) according to the manufacturer's protocol. The auditory cells were homogenized in the TRI reagent and centrifuged at 12000×g for 15 min at 4°C to separate the cell debris. Total RNA was precipitated with 10-µL polyacryl carrier (Molecular Research Center, Ohio, USA). The RNA pellet was then washed with 75% ethanol, dissolved in RNase and DNase-free water (Invitrogen, California, USA), and stored at −80°C until use. Complementary DNA (cDNA) was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen, California, USA). The reaction mix was prepared and its protocol was performed according to the manufacturer's recommendation. Briefly, the reaction cycle was as follows: 10 min at 23°C for primer priming, 60 min at 42°C for reverse transcription, and 10 min at 94°C for enzyme inactivation. The gene expression level was determined by quantitative polymerase chain reaction (qPCR). The genes of interest were the stem-cell markers Sox2 and Rex1, the auditory progenitor cell marker nestin, and the cochlear hair cell-specific genes myosin VIIa and calretinin. Primers were designed with Primer 3 software [16] based on the published GeneBank database sequences (Table 1). The reaction kinetic and specificity of each primer set was verified with standard curve (Ct value versus serial dilution of total RNA) and melting curve profiles. QPCR reaction was performed using iCycler thermocycler (Bio-Rad, California, USA) and data were analyzed using the Bio-Rad iCycler software. Each reaction mixture consisted of iQ SYBR Green Supermix (Bio-Rad, California, USA), forward and reverse primers (400 nM of each), and 2-µl cDNA template. The following PCR condition was used, as reported previously: cycle 1: 95°C for 3 min (1×) and cycle 2: step 1, 95°C for 10 s and step 2, 61°C for 30 s (40×) [17]. Melting curve analysis was performed to check the reaction specificity. Expression levels of target genes were normalized to GAPDH.

Table 1. Polymerase chain reaction (PCR) primers list

Gene	Accession number	Forward Primer	Reverse Primer	Size (bp)
Nestin	NM_016701	GCAACAGAGCAAGATGAGGAC	CCT CTT TTGGTTCCT TTCCAG	232
Sox2	NM_011443	ACTTTTGTCCGAGACCGAGA	CTCCGGGAAGCGTGTACTTA	149
Rex1	NM_009556	GCGGTGTGTACTGTGGTGTC	AGTTTCGAGCTCTCCGTG AA	163
Myosin VIIa	NM_008663	CACTTACATCCCTGACCGTGA	GGGCATAATTGACCACATCCT	146
Calretinin	NM_007586	GGCCCTATGATGAACCTAAGC	CGCTTCCATCCTTGTCATAAA	204

bp: base pair

Immunocytochemistry Analysis

Immunostaining was performed on cells harvested from the basilar membrane at P0 and P3 with auditory progenitor marker [mouse monoclonal anti-nestin (BD, California, USA)] and early development hair cell markers (rabbit monoclonal anti-calretinin and rabbit monoclonal anti-myosin VIIa (Novus Biologica, Colorado, USA). Cells cultured in chamber slides were washed three times with cold tris-buffered saline (TBS) before being fixed with 4% paraformaldehyde for 24 h at 4°C. After fixation, the cells were washed three times with TBS buffer and air-dried for 5 min. Non-specific antigens were blocked for 1 h at 37°C with 10% goat serum (Chemicon, California, USA). Then, the cells were incubated overnight at 4°C with primary antibodies [nestin (1:200), myosin VIIa (1:200), or calretinin (1:1400)] diluted in 1% goat serum. Samples were then rinsed with TBS+0.1% Triton X-100 (Sigma, UK) and incubated for 1 h with secondary antibody labeled with fluorescein isothiocyanate (FITC). Then, the cells were counterstained with DAPI and observed under florescent microscope (Nikon Ti-U, Nikon Inc., Tokyo, Japan).

Statistical Analysis

Growth rate and viability of auditory cells and gene expression level were calculated using data collected from six samples. Gene expression values were normalized between the target genes Sox2, Rex1, nestin, myosin VIIa, and calretinin and the reference gene GAPDH within wells. Values were expressed as the mean expression relative to GAPDH mRNA±standard error of the mean (SEM). Results were analyzed using Student's t-test (Microsoft Excel; Microsoft Corporation, Washington, USA); differences were considered to be significant at $p<0.05$.

RESULTS

Morphological Features of Basilar Membrane Cell Culture

Cells at P0–P3 grown using two different media were morphologically compared (Figure 1a). In GFEM, the cells formed colonies displaying different morphologies. The cells reached 100% confluency at days 9–10. A total of 5.25×10^4 cells per cochlea were obtained at the end of P0. The cells were further passaged with a seeding density of 5000 cell/cm^2. The cells at P1 reached a confluency of 100% in 6–7 days, and most of the cells morphologically changed to a spindle shape.

In BGM, the cells attached to the culture plate only after 3 days. They slowly formed colonies, and the number of colonies was also lesser compared with cells grown in GFEM. The cells showed a spindle-shaped morphology and reached a confluency of 100% on days 11–14. After first passage, the cells became confluent more rapidly, and at P2 and P3, cell populations were dominated by spindle shaped cells.

Growth Kinetic of Basilar Membrane Cells

In term of cell viability, there was no significant difference between the cells cultured in BGM and GFEM, except at passage 3 where the cells cultured in GFEM were significantly more viable compared with the cells cultured in BGM ($p<0.05$) (Figure 1b). Cells cultured in both media demonstrated cell viability of 91%–97% at all passages until P3.

In term of growth rate, the cells grown in GFEM showed significantly higher growth rate than the cells grown in BGM ($p<0.05$) (Figure 1c). The growth rate of cells grown in GFEM was 2.8-fold and 6-fold higher at P1 and P3, respectively, than cells grown in BGM. However, both cell populations show a decrease in growth rate from P1 to P3.

Quantitative Gene Expression of Auditory Cells

The qPCR analysis showed that the cells in both culture mediums expressed the stem-cell markers Sox2 and Rex1, the auditory progenitor cell marker nestin, and the hair cell-specific genes myosin VIIa and calretinin. Expression level of Sox2 by cells grown in BGM showed an irregular trend where the Sox2 expression was slightly reduced from P0 (0.0026±0.0004) to P1 (0.0020±0.0005) before increasing again at P2 (0.0034±0.0008) and P3 (0.0059±0.0008) (Figure 2a). In contrast, Sox2 expression in GFEM increased from P0 to P1 and then continuously reduced in P2 and P3. The expression level of Sox2 is significantly higher ($p<0.05$) in BGM than in GFEM at P2 and P3. The expression of Rex1 by auditory cells in the BGM at P0 was six times higher than that in GFEM. However, the expression level decreased in following passages, and the Rex1 expression by cells in BGM was lower than that by cells in GFEM at P2 and P3; however, the differences were insignificant ($p>0.05$) (Figure 2b).

Nestin expression level gradually increased from initial seeding (0.0022±0.0005) to P2 (0.0044±0.0012) but reduces at P3 (0.0039±0.0008) for cells grown in BGM. Nestin expression by cells cultured in GFEM increased from 0.0028±0.0006 at P0 to 0.0046±0.0009 at P1 but reduced to 0.0033±0.0008 at P2 and 0.0015±0.0001 at P3. There was no significant difference in the expression of nestin by the cells grown in the two media, except at P3 where the cells cultured in BGM showed significantly higher nestin expression (0.0039±0.0008) than cells cultured in GFEM (0.0015±0.0001; $p<0.05$) (Figure 2c).

In contrast, gene expression level of calretinin reduced throughout passaging for both mediums. The cells in BGM expressed higher levels of calretinin after the initial seeding P0 (17.18±2.419 E-05), but the expression level continuously reduced in subsequent passages. The expression of calretinin in GFEM also showed a downward expression trend across passages, and it was lower than that in BGM at all passages. The difference between the groups was only significant at P1 ($p<0.05$) (Figure 2d). Myosin VIIa expression was significantly higher in auditory progenitor cells in the BGM than in GFEM at all passages ($p<0.05$) and showed a downward expression trend across the passages for both the culture media (Figure 2e).

Immunocytochemistry Analysis

Expression of the auditory progenitor cell marker nestin and the hair cell markers calretinin and myosin VIIa on the cells cultured in the two different media were compared at P0 and P3 (Figure 3). The results showed that cells in both groups displayed positive staining for all markers. However, the expression level could not be quantitated as the number of cells differs among the compared culture wells.

DISCUSSION

Since the development of regenerative medicine, many attempts have been made to culture auditory hair cells; however, the cells underwent apoptosis after a week of culture [18, 19]. Following this, many studies focused on culturing the inner ear progenitor cells and other stem cells and attempted to differentiate them into functional hair

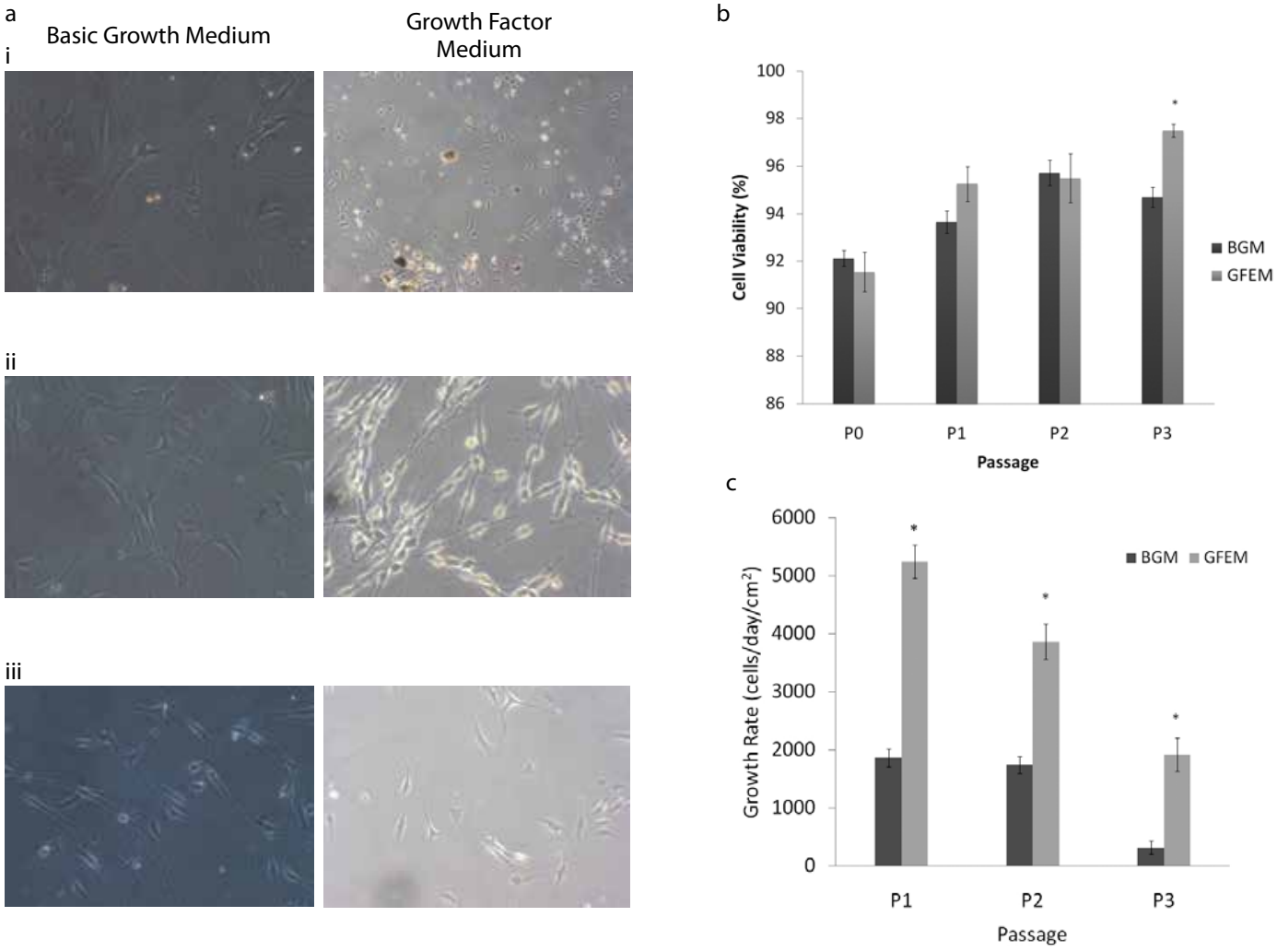

Figure 1. a-c. Morphology of basilar membrane cells at 40× magnifications in basic growth medium (BGM) and in growth factor-enriched medium (GFEM) at passage 1 (a; i. epithelial-like cells, ii. fibroblastic-like cells, and iii. neuron-like cells). Viability of the cells cultured from the cochlear tissue membrane. *p<0.05 at passage 3 (b). Growth kinetic of the cell cultured from basilar membrane. There was significant difference (p<0.05) in the growth rate of cells cultured in BGM and GFEM at all passages (c).

cells [20, 21]. These inner ear stem cells, which are regarded as auditory progenitors, share the properties of progenitor cells during hair cell development and predicted to have a regenerative capacity [21]. There are various strategies and methods by which progenitor cells have been isolated from the neonatal cochlear tissue in the past. Most of them isolated progenitor cells by culturing the cochlear epithelial sheet and maintained the cells in a spherical form [22-24], and some used hair cell expansion to culture them [25]. With the refinement of culture media and in situ techniques, cells from the cochlear epithelial sheet were isolated. We have modified these techniques using different types of enzymes with a lesser time for digestion. Cells were cultured in an adherent condition as they were easy to maintain and observe. To increase cell proliferation, growth factors were being used. Growth factors such as EGF, IGF, BFGF, and N-2® and B-27® supplements (Gibco) were used and reported to enhance the growth of these cells [18, 26]. Auditory cells cultured with the supplementation of the growth factors EGF, IGF, BFGF, and N-2® and B-27® supplements in this study showed a significantly higher proliferation rate than cells without the supplementation of additional growth factors. This is important as the higher cell number will facilitate the successful implantation of these cells for hearing restoration.

Sox2 and Rex1 genes are commonly used to characterize the stem cell population in the cultured cells. Sox2 is a marker of cochlear prosensory cells and persists in cochlear supporting cells. It is one of the earliest markers for inner ear prosensory development [27]. Meanwhile, Rex 1 is a known marker for undifferentiated embryonic stem cells. Cells in GFEM showed a significantly decreased expression of Sox2 at P2 and P3 compared with the expression of Sox2 by cells in BGM. This presumably shows that the multipotency of the cells in GFEM is significantly reduced compared with cells in BGM from P2 onwards. It is noteworthy that the maintenance of high levels of Sox2 expression inhibits hair cell formation, and the downregulation of Sox2 is necessary for hair cell generation [27]. Rex1 expression was significantly lower in cells cultured with GFEM at P0, showing that the cells are losing their stemness properties at initial culture stage when cultured in GFEM. Rex1 at P0 is significantly higher in BGM than in any other condition. This is probably because of augmentation by Sox2 genes, which transactivate Rex1 promoter [28]. Rex1 expression at P0 was higher in BGM than in any other condition. This could be because of the downregulation of Rex1 by growth factors in GFEM grown cells.

Growth factor supplementation also seems to reduce the nestin expression significantly at passage P3 compared with cells grown in

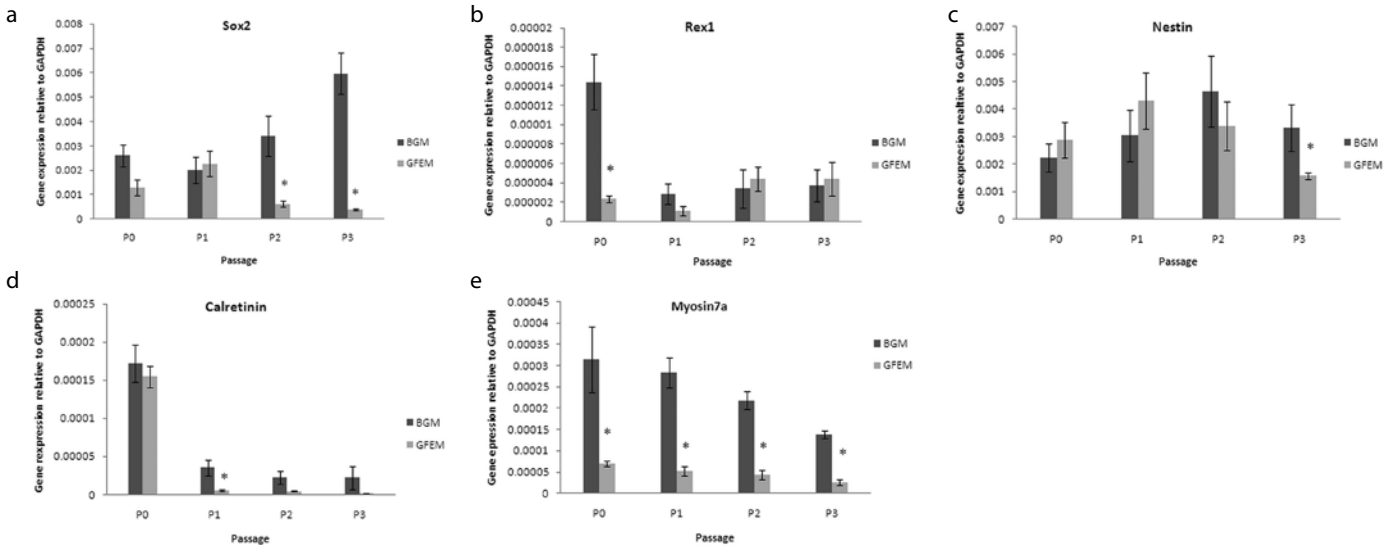

Figure 2. a-e. Gene expression analysis of the stem-cell markers Sox2 (a) and Rex1 (b); the auditory progenitor cell marker nestin (c); the cochlear-specific hair cell genes myosin VIIa (d) and calretinin (e) for cells cultured in basic growth medium (BGM) and in growth factor enriched medium (GFEM). Data were expressed as mean±SEM (n=6; *p<0.05).

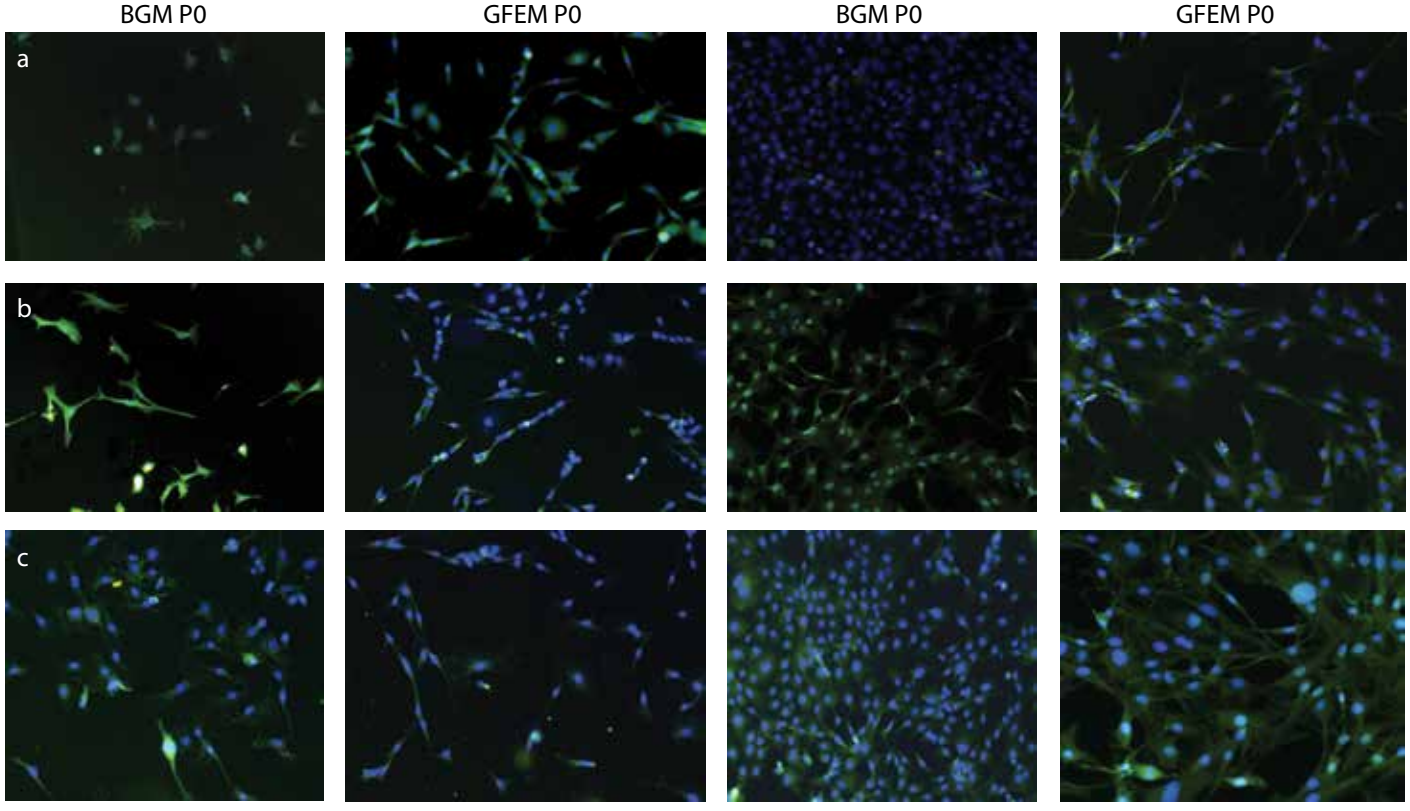

Figure 3. a-c. Immunostaining of the auditory progenitor markers nestin (a) and the hair cell-specific markers calretinin (b) and myosin VIIa (c). The target protein expression was detected by green fluorescence. Cell nuclei were counterstained with DAPI (blue; 40× magnification). No difference was observed in the expression at passage 0 and passage 3 among the cells grown in basic growth medium (BGM) and in growth factor-enriched medium (GFEM).

BGM. Nestin-positive cells, which are regarded as neural progenitor cells, have been found to be a good source for new hair cell formation [29]. Nestin expression also was found to be increased in damaged cochlear [30]. In our study, we have shown that the presence of nestin throughout the culture is noteworthy in the regeneration of hair cells with or without the supplementation of growth factors for a maximum culture period of 3 weeks. Myosin VIIa and calretinin are important genes responsible for developing and characterizing hair cells, whereas calretinin is a calcium-binding protein for the functioning of hair cells [31]. The gene expression analysis in this study showed that the cells supplemented with growth factors began to lose their hair cell-specific gene expression. Both the myosin VIIa and calretinin genes were significantly reduced in cells supplemented with growth factors. Myosin expression was 4–5 folds lower in cells supplement-

ed with growth factors than in cells without the supplementation of any growth factors. This shows the added growth factors in cohort or individually inhibiting the expression of myosin genes by the inner cells of the cochlea.

The limitation of the study included that the addition of growth factors into the BGM was carried out in a concoction, and they were not studied for the individual effect on the basilar membrane cells. As the concoction of the growth factor used in this study showed a downregulation of the hair cell-specific markers, the individual effect of these growth factors can shed light on the growth factor selection for the upregulation of those markers. However, the number of cells obtained from the cochlea of each mouse is very limited; therefore, the study with individual growth factors was hampered during the period of this study.

Regeneration of cochlear hair cell is still a major challenge to researchers. The use of various types of growth factors are known to induce cell proliferation and thought to enhance the possibility of increasing the hair cells phenotype. Ultimately, the regenerated hair cells can be used to treat the damaged auditory tissue and potentially reverse hearing loss. However, in this study, we found that this combination of growth factor supplementation could not maintain the phenotype of cells obtained from the cochlear membrane. Although the growth factor increases the growth kinetic of cells, it does not increase the hair cell-specific genes. There is also a possibility of contamination with hair cells at initial passage. Thus, as the hair cells degenerate through the passages, the hair cell-specific genes show a decreasing trend.

The growth factors EGF, IGF, BFGF, and N-2® and B-27® supplements cumulatively caused the downregulation of the hair cell-specific markers obtained from the basilar membrane. More specific doses and combinations need to be further investigated as these specialized cells are not easy to be maintained under culture condition.

Ethics Committee Approval: This study was approved by the Universiti Kebangsaan Malaysia Research and Ethical Committee (UKM 1.5.3.5/244/SP-P/02-01-02-SF0441).

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