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BONE MARROW MESENCHYMAL STEM CELLS ARE PROGENITORS IN VITRO FOR INNER EAR HAIR CELLS

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Abstract

Stem cells have been demonstrated in the inner ear but they do not spontaneously divide to replace damaged sensory cells. Mesenchymal stem cells (MSC) from bone marrow have been reported to differentiate into multiple lineages including neurons, and we therefore asked whether MSCs could generate sensory cells. Overexpression of the prosensory transcription factor, *Math1*, in sensory epithelial precursor cells induced expression of *myosin VIIa*, *espin*, *Brn3c*, *p27Kip*, and *jagged2*, indicating differentiation to inner ear sensory cells. Some of the cells displayed F-actin positive protrusions in the morphology characteristic of hair cell stereociliary bundles. Hair cell markers were also induced by culture of mouse MSC-derived cells in contact with embryonic chick inner ear cells, and this induction was not due to a cell fusion event, because the chick hair cells could be identified with a chick-specific antibody and chick and mouse antigens were never found in the same cell.

Stem cells resident in bone marrow are the source of blood cells, but in addition to these hematopoietic stem cells, the bone marrow contains mesenchymal stem cells (MSCs) that can differentiate into cell types of all three embryonic germ layers (Colter et al., 2000; Doyonnas et al., 2004; Herzog et al., 2003; Hess et al., 2003; Jiang et al., 2002; Pittenger et al., 1999). This has been demonstrated *in vivo* in studies that track transplanted bone marrow cells to specific tissues where they differentiate into the resident tissue type (Mezey et al., 2003; Weimann et al., 2003). Differentiation may occur in part due to cell fusion (Wang et al., 2003; Weimann et al., 2003) in which the bone marrow cell takes on the identity of the peripheral cell. Recent studies have documented *ex vivo* differentiation of bone marrow derived stem cells into muscle cells (Doyonnas et al., 2004), cartilage (Pittenger et al., 1999), insulin-producing cells (Hess et al., 2003), and neurons (Dezawa et al., 2004; Hermann et al., 2004; Jiang et al., 2003; Kicic et al., 2003), both *in vitro* and after injection of these cells *in vivo*. Many of these cells have been used for transplantation and are a

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preferred source of new cells for therapies because the transplanted cells are immunologically matched when harvested from a patient to be treated and because they have been extensively used in clinical applications so that their safety is known. Because sensory cells reside in the neural lineage as demonstrated in the ear by lineage analysis in the chick (Sato and Fekete, 2005), and by the effect on hair cell number of disruption of neurosensory progenitors (Ma et al., 2000), differentiation of bone marrow stem cells to neurons raises the possibility that sensory cells could also be produced from bone marrow cells.

A source of sensory cells and neurons for regeneration of inner ear cells would provide a valuable tool for clinical application because neurons and hair cells could eventually be employed in cell replacement therapy for hearing loss. Recent work has shown that hair cells and neurons can be differentiated from endogenous stem cells of the inner ear (Li et al., 2003a; Rask-Andersen et al., 2005) and other work has shown that endogenous cells of the sensory epithelium can be converted to hair cells when the proneural transcription factor, Math1, is expressed exogenously (Izumikawa et al., 2005; Zheng and Gao, 2000) and yet the endogenous stem cells of the inner ear do not spontaneously generate hair cells. Injection of whole bone marrow to reconstitute a lethally irradiated mouse resulted in engraftment of these cells in areas occupied by inner ear mesenchymal cells and fibrocytes but did not yield hair cells (Lang et al., 2006). We therefore asked if we could steer adult bone marrow cells to hair cells.

By a combination of growth factor stimulation and expression of the transcription factor, Math1, that is required for hair cell formation in the inner ear, we show here that MSCs derived from bone marrow can be induced to differentiate into hair cells. The neurosensory progenitors obtained from bone marrow can be converted to sensory cells by co-culture with cells of the developing sensory epithelium, moreover, even in the absence of Math1 expression.

RESULTS

Sensory progenitors from mesenchymal stem cells

We obtained mesenchymal stem cells from mouse bone marrow by culturing adherent cells from the marrow under high serum conditions. The MSCs were negative for CD34 and CD45, markers for hematopoietic stem cells in bone marrow (Jiang et al., 2002; Pittenger et al., 1999) and positive for CD44 and Sca-1, markers for MSCs (Dezawa et al., 2004). Sca-1 was present on 5.2% of the cells and CD44 was present on 11.5% of the cells based on immunohistochemistry and the percentages determined by flow cytometry were similar (Fig. 1A and 1D and Table I). We detected co-expression of CD44 and nestin as well as Sca-1 and nestin on a small percentage of the cells (Fig. 1B and 1C). We confirmed the previously reported capacity of MSCs to be converted to chondrocytes (Pittenger et al., 1999) and neurons (Dezawa et al., 2004). Their potential to differentiate into chondrocytes that expressed type II collagen (Fig. 1E) was demonstrated by incubation in medium containing TGF- β , insulin and transferrin. Removal of serum and culture of the MSCs for 21 days in medium containing neuronal growth supplements resulted in differentiation to neurons (Dezawa et al., 2004) as shown by neuronal markers (Fig. 1F).

To determine whether otic vesicle growth factors that are important in the early development of inner ear progenitor cells could have a similar effect on MSCs, we removed the serum from the MSCs after 3-5 passages and cultured the cells in serum-free medium containing IGF-1, EGF and bFGF. When we assessed the expression of neural progenitor cell markers in the resulting cultures, we found that *Otx2*, *nestin*, *Sox2*, and *Musashi* were expressed in increased amounts in these cells, which we subsequently refer to as progenitor cells, relative

to MSCs based on RT-PCR (Fig. 2A). *Pax6* was found in the progenitor cells but not in the MSCs (Fig. 2A). *Pax2* was not expressed. A low level of *Pax5* was detected but *Pax8* was not expressed (data not shown). A similar pattern of expression was seen for the stem cell marker, *Oct4*, which was expressed in the progenitor cells but interestingly, given its role in maintaining the pluripotency of stem cells, was not found in the MSCs. The increase in expression of *nestin* in the progenitor cells relative to the MSCs (Fig. 2A) was confirmed by immunohistochemistry (Fig. 2B and 2C and Table I) and was significant ($p < 0.001$). Additional markers of the hair cell and neural lineages (*Math1*, *Brn3c*, *GATA3*) and neuronal markers (*TrkB* and *TrkC*) were also expressed in the progenitors (Fig. 2A).

Because of the expression of *TrkB* and *TrkC* in the progenitor cell populations, we tested whether incubation with NT-3 and BDNF, the neurotrophins that bind to these receptors, would increase the yield of progenitor cells or alter the expression of genes for hair cell or neuronal fate. We found an increase in expression of *Otx2*, *Sox2*, *nestin*, and *Musashi* under these conditions as well as an increase in *Oct4* expression (Fig. 3A), indicating that the cells may have adopted a neural progenitor cell fate. The neurotrophin-mediated conversion to progenitor cells had a more rapid time course that we found for EGF, IGF-1 and bFGF alone. The expression of proneural transcription factors, *NeuroD* and *Ngn1*, as well as neural and hair cell lineage markers, *GATA3*, *Math1* and *Brn3c*, were also increased and the expression of *Ngn1* and *NeuroD*, which select for a neural over a hair cell fate in the inner ear (Kim et al., 2001; Matei et al., 2005) were higher when NT-3 and BDNF were included in the differentiation medium. Other transcription factors expressed in the otic precursors during development, *Zic2* and *Pax6*, were elevated in the progenitor cells relative to the MSCs, and *Zic1* expression was not observed. This suggests that NT-3 and BDNF induced the formation of cells of a neural lineage that were potentially destined to become both neurons and hair cells. However, the cells were not converted to hair cells or neurons because markers for these cells were not found (Fig. 3A, hair cell markers myosin VIIa and *espin*). We also tested for the expression of genes characteristic of other epithelial cells in the cochlea such as supporting cells, because the progenitors for hair cells can include or give rise to these cells and found that the progenitors expressed *S100A*, *p75^{Trk}*, *claudin 14*, *connexin 26*, and *Notch1*.

Transfection with a *Math1* expression plasmid converts progenitors to hair cells

To test whether the progenitor cells could act as inner ear precursor cells, we evaluated whether overexpression of *Math1*, a transcription factor that is known to push competent progenitors to a hair cell fate (Izumikawa et al., 2005; Zheng and Gao, 2000), would increase the expression of hair cell markers. The efficiency of *Math1* transfection was tested by counting green fluorescent cells after transfection with a vector coding for GFP expression in addition to *Math1*. When MSCs were transfected, as many as 2% of the cells were GFP positive at 24 h (Fig. 4A). RT-PCR at day 14 showed that the transfected cell population expressed markers of developing sensory epithelia, such as *p27Kip*, *Brn3c* and *jagged2*, and mature hair cells markers, *myosin VIIa* and *espin* (Fig. 4B) as well as increased expression of *Ngn1* and *NeuroD*. We also detected expression of supporting cell markers, *S100A*, *p75^{Trk}*, *claudin 14*, *connexin 26*, and *Notch1*, indicating that the progenitor cells could give rise to hair cells and supporting cells (Fig. 4C). Selection of MSC transfectants with stable *Math1* expression increased the percentage of GFP-positive cells (Fig. 4D). Incubation of these cells in the growth factors described above followed by immunohistochemistry yielded cells with expression of *Math1* and myosin VIIa respectively in 7.7% and 7.1% of the total cells (Fig. 4E). Differentiation under growth factor stimulation gave rise to cells with *Brn3c* in the nucleus and myosin VIIa in the cytoplasm (Fig. 4F). These cells were positive for both markers in the same cells, with 92% of the *Math1*-positive cells showing staining for myosin VIIa, and 77% of the *Brn3c*-positive cells showing

staining for myosin VIIa. Examination of the myosin VIIa positive cells for F-actin (Fig. 4G and H) indicated that some of the cells (4.9% of the myosin VIIa-positive cells) had developed protrusions at their apical poles. These protrusion had the polarized appearance of stereociliary bundles and were positive for espin (Fig. 4G).

Conversion of progenitors to hair cells is stimulated by developing otocyst cells

To test whether the developing otocyst produced factors that would increase the differentiation of MSCs to hair cells, we performed co-culture experiments of E3 chick otocyst cells with MSCs. After culture in the presence of the chick otocyst cells for 21 days, we found increased expression of *myosin VIIa*, *jagged2*, *p27Kip*, *Brn3c* and *Math1* by RT-PCR (Fig. 5A). The factor(s) was unlikely to be a secreted molecule because fixation of the cells did not diminish their ability to promote differentiation after exposure for 14 days, while conditioned medium was ineffective in 14 days (Fig. 5A). Conversion of the stem cells to hair cells could be followed by appearance of green fluorescence in the cultures using MSCs derived from transgenic *Math1-nGFP* mice that express a nuclear version of enhanced GFP when *Math1* enhancer elements are activated (Chen et al., 2002; Lumpkin et al., 2003). These green cells were observed in the co-cultures with chick otocyst cells (Fig. 5B) and the cells were co-labeled with antibody to myosin VIIa.

When the progenitor cells were injected into chick otocysts obtained at E3, conversion of progenitors to cells with hair cell properties (5% of the myosin VIIa-positive cells were positive for nGFP) was observed (Fig. 6A). The murine hair cells were seen to incorporate into the hair cell bearing epithelia of the developing chicken otocyst as detected by expression of GFP (Fig. 6B). One possible explanation for the expression of hair cell genes by the MSC-derived cells in co-culture is fusion with chick cells. To rule this out we labeled the cells with an antibody to chick hair cell antigen (Bartolami et al., 1991). Native chick hair cells could be detected lining the internal cavity of the otocyst (51% of 1,352 cells from 15 otocyst injections that stained for myosin VIIa were positive for chick hair cell antigen), and the cells that expressed nGFP and hair cell markers did not co-express chick hair cell antigen (Fig. 6C) and were therefore of mouse origin and not the product of cell fusion.

DISCUSSION

Stem cells in bone marrow are known to be the precursors for all lymphoid and erythroid cells, but mesenchymal stem cells in bone marrow also act as precursors to bone, cartilage, and fat cells (Colter et al., 2000; Pittenger et al., 1999). In addition to mesenchymal tissues, these stem cells have been shown to give rise to cells of other lineages including pancreatic cells (Hess et al., 2003), muscle cells (Doyonnas et al., 2004) and neurons (Dezawa et al., 2004; Hermann et al., 2004; Jiang et al., 2003). In this study we have extended the range of cell fates available for these bone marrow-derived cells to include cells of the neurosensory lineage and we have demonstrated their differentiation to inner ear hair cells.

Expression of *Math1* in the inner ear progenitor cells was sufficient to drive them into adopting hair cell markers. Studies of *Math1* expression in the ear have indicated that this helix-loop-helix transcription factor occupies a key place in the hierarchy of inner ear transcription factors for differentiation of hair cells. The progenitor cells also responded to contact with developing otocyst cells from the chicken embryo by differentiation to sensory epithelial cells without the requirement for exogenous *Math1* as was evident by nGFP expression from the *Math1* enhancer and co-expression of myosin VIIa after co-culture and differentiation. Neurons that express markers of sensory cells have been induced from bone marrow MSCs in previous work by incubation with otocyst and hindbrain-conditioned medium (Kondo et al., 2005) from embryonic mice.

Experiments performed in an attempt to understand how contact of the MSCs with developing otocyst cells provided a signal that induced their differentiation to hair cells demonstrated that the inductive effect was through a cell surface molecule as opposed to a secreted factor. Injection of the MSC into the developing otocyst *in vitro* indicated that hair cells that differentiated from the stem cells were integrated into the chick otocyst epithelium, demonstrating that the environment provided by developing chicken otocyst cells could guide differentiation and integration of suitable progenitor cells. The instructive influence has also been seen previously with inner ear-derived stem cells and murine ES cell-derived progenitor cells (Li et al., 2004a; Li et al., 2003a; Li et al., 2003b). The effect of the co-incubation with otocyst cells may be simply to activate *Math1* expression and a sufficient amount of *Math1* may be required to allow hair cell differentiation since the MSCs had low levels of *Math1* but did not have detectable sensory epithelial cell markers. This type of high level expression could be needed for *Math1* to overcome the level of preexisting endogenous inhibitors that interact with *Math1* protein. The murine cells could be clearly distinguished from the chick hair cells that differentiated at the same time by their expression of nGFP and by immunolabeling of the chick hair cells with a species-specific antibody. The cells were never co-stained (based on examination of 1,352 cells), indicating that the mouse hair cells had differentiated from stem cells and did not arise from cell fusion. The observation of supporting cell markers from the MSC-derived progenitor cells after growth factor induction may be correlated to their origin from a common progenitor during *in vivo* development (Matei et al., 2005; Satoh and Fekete, 2005). Since hair cells can be induced to develop from supporting cells after introduction of the *Math1* gene (Izumikawa et al., 2005; Zheng and Gao, 2000), the role of supporting cells as potential progenitors for hair cells via transdifferentiation has been discussed (Izumikawa et al., 2005). The expression of supporting cell genes may reflect an intermediate or accompanying stage on the way to becoming hair cells; in *Math1* knockout mice undifferentiated cells with markers of supporting cells have been observed to activate the *Math1* gene (Fritzsche et al., 2005; Woods et al., 2004). Alternatively, supporting cells could be induced by the developing hair cells: ectopic hair cells in the greater epithelial ridge induced supporting cell markers in surrounding cells (Woods et al., 2004). The MSCs could be induced to become hair cell progenitors by bFGF, EGF and IGF-1, factors that potentially stimulate the *in vivo* formation of these progenitors (Leon et al., 1995; Pauley et al., 2003; Zheng et al., 1997), and these progenitors were able to give rise to hair cells after overexpression of *Math1*. An increase in expression of neural progenitor markers could be caused by expansion of the cells that express these markers or by differentiation of MSCs to the neural progenitor phenotype.

Math1 expression led to strong expression of helix-loop-helix transcription factors, *Ngn1* and *NeuroD*. Several previous studies have indicated that *Math1* expression can increase these transcription factors. In mouse cerebellum *Math1* expression leads to overexpression of *NeuroD* (Helms et al., 2001). In zebrafish *NeuroD* is not expressed in the absence of *Math1* (Sarrazin et al., 2006) and is required for hair cell formation. The related mouse achaete-scute (*Mash1*) upregulates *Ngn1* (Cau et al., 1997). However, *Ngn1* was downregulated by overexpression of *Math1* in chick neural tube (Gowan et al., 2001).

We found that MSC-derived progenitor cells expressed neurotrophin receptors. BDNF and NT-3 play important roles in maturation of inner ear neurons (Fritzsche et al., 1997; Pirvola and Ylikoski, 2003), and in differentiation of neural stem cells to neurons (Ito et al., 2003), and we therefore tested whether the fate of the progenitors could be modulated by neurotrophins. Incubation with these factors resulted in enrichment of progenitors that could be converted to hair cells by subsequent *Math1* overexpression (Izumikawa et al., 2005; Zheng and Gao, 2000) or co-culture with chick otocyst cells. Since NT-3 and BDNF were found to increase both *Math1* expression and differentiation in neural stem cells (Ito et al.,

2003) neurotrophins could directly increase differentiation of MSCs or could increase their competence to respond to overexpressed *Math1*.

Analysis of the progenitor cells obtained from the MSCs revealed parallels with natural development of the inner ear sensory epithelia. The MSC-derived progenitors expressed *Sox2*, which must be present for subsequent hair cell differentiation in the developing otocyst (Kiernan et al., 2005). The expression of *Math1* in cells that did not have myosin VIIa and the appearance of myosin VIIa at later time points is consistent with the order of their expression during development based on immunohistochemistry (Chen et al., 2002). The lack of *Pax2* expression was surprising since the paired box transcription factor is ubiquitously expressed in the otocyst (Burton et al., 2004; Li et al., 2004b). This may suggest that *Pax2* is not required or that it can be replaced by another factor for the conversion of MSCs to hair cells. *Pax5* was detected and may substitute for *Pax2* based on their functional equivalence (Bouchard et al., 2000). This is consistent with the analysis of the *Pax2* null mouse (Burton et al., 2004), which appears to develop hair cells despite severe disruption of the normal morphology of the cochlea. The lack of *Zic1* expression relative to *Zic2* is also found during development of a hair cell phenotype as compared to sensory neurons in the otocyst (Warner et al., 2003) and is thus consistent with the development of a hair cell phenotype. The identification of inductive molecules on chick otocyst cells that are not present in conditioned media will provide further insights into hair cell differentiation.

The isolation of progenitor cells that can give rise to the tissue of origin, as observed in the inner ear (Li et al., 2004a; Li et al., 2003a), might be predicted and yet the cells do not regenerate after damage, possibly because of the decrease in number of inner ear stem cells after birth (Oshima et al., 2006). Therefore, a source of cells to provide replacements for these sensory cells is highly desirable. The *in vivo* role of MSCs in regeneration generally remains uncertain although bone marrow could act as a source of new cells in organs with few progenitors. Despite the demonstration that cells from bone marrow migrate into the brain and heart in adults (Mezey et al., 2003; Weimann et al., 2003) and differentiate into neurons in the brain, hematopoietic stem cells from bone marrow were not converted to cardiomyocytes after injection (Murry et al., 2004) and conversion to neurons was extremely rare (Wagers et al., 2002; Weimann et al., 2003). The most successful attempts at regeneration by adult stem cells from other tissues have been obtained after a lesion (Doyonnas et al., 2004; Edge, 2000; Hess et al., 2003; Pagani et al., 2003) and tissue damage may be required to see cell replacement by bone marrow-derived cells. Whether bone marrow-derived cells play any regenerative role in the sensory or peripheral nervous system in a spontaneous response to damage *in vivo* is an unanswered question, but, although low-level replacement of hair cells by bone marrow cells *in vivo* cannot be ruled out, spontaneous replacement of sensory cells is unlikely to be significant given the lack of hair cell regeneration seen in the adult cochlear and vestibular systems (Hawkins and Lovett, 2004; White et al., 2006).

Although stem cells are present in the inner ear (Li et al., 2004a; Li et al., 2003a; Rask-Andersen et al., 2005), hair cells do not regenerate after damage, and, therefore, a source of cells that could potentially be used for cell transplantation in a therapeutic replacement of these sensory cells has important implications for treatment of sensorineural hearing loss. Bone marrow has been harvested and used extensively in clinical applications and is a highly desirable source, because cells from a patient's bone marrow could potentially be transplanted without the problem of immune rejection. Depending on the feasibility of many additional steps such as surgical approaches for cell transplantation into damaged cochleae, a treatment regimen for hearing loss may become possible.

METHODS

Bone marrow stem cell isolation, culture, characterization

Cells were obtained from bilateral femurs and tibias of 4 week old C57BL/6 or *Math1-nGFP* mice (a gift from Jane Johnson (Helms et al., 2000)) by flushing out the bone marrow with MEM- α (Gibco/BRL) containing 10% fetal bovine serum (FBS; BioWhittaker, Cambrex, NY) and 1 mM glutamine (Gibco/BRL). Pelleted cells were resuspended and mixed with RBC lysis buffer (Gibco/BRL). Approximately 5×10^6 cells were cultured on a 10 cm dish overnight in MEM- α with 9% horse serum, 9% FBS, 1% Gluta-Max (Invitrogen) and 100 units/ml penicillin and streptomycin (100 μ g/ml, Sigma) at 37°C in a 5% CO₂ atmosphere. Nonadherent hematopoietic stem cells were removed, leaving adherent bone marrow stromal cells. When the cells became confluent, trypsinization was performed and the cells were cultured and passaged three to five times, with media changes every 3-4 days. These cells are referred to as mesenchymal stem cells (MSC).

For chondrogenic differentiation, MSC were formed into a micropellet and cultured in DMEM with 10ng/ml TGF β 1, 6.25 μ g/ml transferrin and 6.25 μ g/ml insulin for 2 weeks. For neuronal differentiation, MSC were cultured in DMEM/F12 1:1 containing N2/B27 supplement with bFGF (10 ng/ml) for 14 days and for 7 days without bFGF.

Progenitor cell induction and *in vitro* differentiation

For the induction of progenitor cells, passage 3-5 MSC were trypsinized and transferred to 6-well plates or 4 well plates (BD Bioscience) coated with poly-L-ornithine and gelatin or fibronectin (Sigma) at 5×10^4 cells/ml. Cells were cultured for 5-7 days, and then cultured in serum-free medium composed of DMEM/F12 1:1 containing N2/B27 supplements (Invitrogen). For progenitor cell induction, we used a combination of EGF (20 ng/ml) and IGF-1 (50 ng/ml; R&D Systems, Minneapolis, MN) for 2 weeks followed by the addition of bFGF (10 ng/ml) plus the other growth factors for an additional 2 weeks or a combination of NT3 (30 ng/ml) and bFGF (10 ng/ml) for 4-5 days followed by NT3 (30 ng/ml) and BDNF (10 ng/ml) for 7 days.

Math1 overexpression

We constructed a vector containing the *Math1* coding sequence under EF1 α -promotor control in the pTracer-EF vector (Invitrogen) that has a GFP-Zeocin fusion sequence under the CMVpromoter. Gene transfection was done in the progenitor cell state or as MSC using lipofectamine (Sigma). Cells were cultured in Zeocin (Invitrogen) to obtain stable transfectants. Transfected MSC were cultured in the serum-free conditions with combinations of growth factors.

Culture of MSC with chick otocyst cells

Embryos of the white leghorn strain (Charles River) were harvested 72 hours after placing fertilized eggs onto rocking platforms in a humidified incubator maintained at 38°C. The dissection of otocysts from the extracted embryos was done in cooled PBS, pH7.2, after removal of periotic mesenchymal tissues. The otocysts were trypsinized and dissociated to single cells for plating and 2×10^4 cells were cultured overnight in 4-well plates in 10% FBS. One day after plating, the otocyst cells were fixed with 4% paraformaldehyde for 20 min, or inactivated with mitomycin C (10 μ g/ml) for 3 hours, then washed 4 times with PBS. Conditioned medium from the cultured cells was collected and frozen prior to use on progenitors cells. Progenitor cells (5×10^4 cells/ml) induced in serum-free medium with growth factors, were overlaid on the chick otocyst cells and cultured for 5-7 days with EGF/IGF-1, followed by 10 days with EGF/ bFGF/IGF-1 and withdrawal of growth factors for 5-10 more days. The cells were analyzed by RT-PCR or immunohistochemistry.

Ex vivo chick otocyst injection of progenitor cells

The otocyst from E3 chick embryos were used for injection of progenitor cells. The dissected otocysts were transferred into 7 ml of serum-free DMEM/F12 1:1 containing N2 and B27 on a gelatin-coated tissue culture dish. After attachment of intact otocysts, progenitor cells from MSC (5×10^7 cells/ml) were injected into the otocyst with a micropipette in 2 μ l of medium. The left otic vesicles did not receive cell grafts and served as controls. The otocysts were harvested after 10-14 days, fixed 30 min in paraformaldehyde (4% in PBS), cryoprotected overnight in sucrose (30% in PBS), embedded in TissueTek (EMS) and serially sectioned (16 μ m) with a cryostat (CM3050, Leica, Nussloch, Germany).

Semiquantitative RT-PCR

Total RNA was extracted with the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For reverse transcription, 6 μ g of total RNA was used with SuperScript III transcriptase (Invitrogen) and oligo-dT primers. The PCR cycling conditions were optimized in pilot experiments. Specific cycling parameters were: initial denaturation step at 94°C for 2 min, denaturation 94 °C for 30 s, annealing temperature optimized between 56-60 °C for 30 s, extension 72 °C for 60 s, extension 72°C for 60 s, and followed by 7 min of terminal extension at 72 °C after the last cycle. The number of cycles was optimized between 30 and 35, and conditions were kept constant for each primer. The presented data are from experiments repeated at least 5 times. Control PCR without reverse transcriptase did not produce specific bands. The primer pairs and cDNA product lengths are given in the Supplement.

Flow cytometry

MSC were incubated with antibodies to CD34, CD44, CD45 or Sca-1 (BD Biosciences) and further incubated with secondary anti-mouse antibody conjugated to TRITC. Data were acquired and analyzed using an Agilent 2100 Bioanalyzer system and flow cytometry chips (Agilent Technology Inc., Palo Alto, CA). The reference window was set so that fluorescence from the secondary antibody alone was less than 2%.

Immunohistochemistry

Cells were fixed for 10 min with 4% paraformaldehyde in PBS. Immunostaining was initiated by rehydrating and blocking the sections for 1 h with 0.1% Triton X-100 in PBS supplemented with 1% BSA and 5% goat serum (PBT1). Fixed and permeabilized cells or rehydrated sections were incubated overnight in PBT1. CD34, CD44, CD45, Sca-1 antibodies (BD Biosciences) diluted 1: 40 were used for the characterization of extracted bone marrow cells. Hair cells and bone marrow progenitors were characterized using monoclonal antibody to chick hair cell specific antigen diluted 1:500 (gift from Guy Richardson (Bartolami et al., 1991)); polyclonal antibody to myosin VIIa, 1:500 (Oshima et al., 2006); monoclonal antibody to nestin, 1,000 (Developmental Studies Hybridoma Bank, Iowa City, IA); polyclonal antibody to parvalbumin 3, 1:2,000 (Heller et al., 2002); monoclonal antibody to Math1, 1:100 (Developmental Studies Hybridoma Bank); monoclonal antibody to neurofilament M, 1:200 (Chemicon); Polyclonal antibody to collagen type II, 1:40 (Chemicon); polyclonal antibody to Brn3c (Covance, Princeton); Cy-5 conjugated F-actin 1:1000 (Molecular probe). Samples were washed three times for 20 min each with PBS. Anti-rabbit, anti-guinea pig and anti-mouse secondary antibodies conjugated with FITC-, TRITC-, and Cy-5- (Jackson ImmunoResearch) were used to detect primary antibodies. The samples were counterstained with DAPI for 10 min (Vector Laboratories) and viewed by epifluorescence microscopy (Axioskop 2 Mot Axiocam, Zeiss) or confocal microscopy (TCS, Leica). The counting of immunopositive cells was performed by counting

300 cells in 20 randomly selected microscopic fields and significance was calculated by Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification of MSC markers and multilineage potential by differentiation to known cell types

A. Bone marrow MSCs from passage 3 were immunostained with antibodies against CD44, CD45, CD34 and Sca-1 followed by secondary antibodies against mouse immunoglobulins labeled with TRITC (shown in red). Staining for CD34 and CD45 was negative, but CD44 and Sca-1 were expressed. Nuclei were stained with DAPI (blue). B. Immunolabeling for CD44 and nestin (shown in green). The merged image on the right shows co-staining of a population of cells with both markers. C. Co-expression of nestin and Sca-1. Merged images show co-staining. D. Analysis of bone marrow MSCs by chip flow cytometry indicates the ratio of immunopositive cells for each of these antibodies. E. Potential for lineage differentiation was shown by formation of chondrocytes and extracellular matrix after treatment of bone marrow MSCs with TGF- β . The cells that grew out from a micro-aggregate (top) were stained for type II collagen (bottom). F. Demonstration of lineage potential of bone marrow MSCs to neurons by differentiation in serum-free medium containing neuronal growth supplements and bFGF. Staining for neurofilament (NF-M) is shown in these cells.

Figure 2. Induction of bone marrow MSCs by stimulation with growth factors in serum-free medium

A. The MSCs were treated with IGF-1, EGF and bFGF for 14 days followed by analysis for neural progenitor markers by RT-PCR. MSC (bone marrow MSCs), NP (neural progenitors at 2 wks after induction of progenitor formation). The genes analyzed are shown to the left of the gel. B. Expression of the neural progenitor marker, nestin, was visualized by immunohistochemistry using a secondary antibody labeled with FITC (shown in green). Scale bars are 50 μ m.

Figure 3. Induction of neural progenitors by incubation of MSCs with trophic factors

A. Precursor cells incubated in NT3, FGF and BDNF that support neuronal and sensory cell progenitors in the inner ear were seen by RT-PCR to have gene profiles that included expression of *Oct4*, *nestin*, *Otx2*, and *Musashi*, as well as proneural transcription factors, *GATA3*, *NeuroD*, *Ngn1*, *Math1*, *Brn3c*, and *Zic2*. These cells did not express hair cells genes, *myosin VIIa* and *espin*. B. In the cells obtained after induction with NT3, FGF, and BDNF, genes characteristic of supporting cells (*claudin14*, *connexin 26*, *p75^{Trk}*, *Notch 1*, and *S100A*) were also observed. These progenitor cells thus had expression profiles characteristic of neuronal or sensory progenitors. Genes analyzed are shown to the left of the gels.

Figure 4. Overexpression of *Math1* induced expression of hair cell specific genes

A. Exogenous expression of *Math1* in bone marrow MSCs resulted in green cells and nuclei due to the expression of GFP from the vector. B. Expression of *Math1* followed by treatment of the cells with NT3, FGF and BDNF, gave rise to progenitor cells that subsequently matured into cells expressing hair cell genes, including *espin*, *myosin VIIa*, *jagged 2*, and *Brn3c*, and *p27Kip*, in addition to the proneural genes, *Ngn1* and *NeuroD*. C. The cells under these differentiating conditions also expressed *S100A*, *p75^{Trk}*, *claudin 14*, *connexin 26*, and *Notch1*, consistent with some cells having a supporting cell phenotype. D. MSC cell line selected in Zeocin had a high percentage of GFP expression when cultured in serum. E. After differentiation, the number of hair cell-like cells per DAPI nucleus rose and these cells stained for myosin VIIa (shown in red) and Math1 (shown in green; arrows). F. *Math1* expressing cell line differentiated to cells with nuclei that were immunopositive for Brn3c (green; arrowheads) and cytoplasm positive for myosin VIIa (red; arrows). Nuclei were stained with DAPI (blue). G. The differentiated cells were positive for both espin and F-actin in protrusions at the apex of the cell (arrow). Scale bars are 25 μ m.

Figure 5. Increase in hair cell gene expression after co-culture with chick otocyst cells
 A. The bone marrow MSC derived progenitors were co-cultured for 21 days with chick otocyst cells that had been treated with mitomycin C (Mito C) and the expression of *jagged 2*, *p27Kip*, *Math1*, *Brn3c*, *myosin VIIa* and *espin* was increased, whereas the expression of these genes in chick cells was undetectable. Chick otocyst cells that had been fixed by incubation with paraformaldehyde were less effective (PFA) than the unfixed cells but did cause differentiation of the progenitors. Conditioned medium from the chick cells (Cnd Med) had no effect (levels of expression of these markers similar to previously shown data for differentiating conditions). B. Expression of *Math1* and myosin VIIa in cells from a *Math1-GFP* mouse showed green fluorescence corresponding to the induction of this marker in the nucleus and had expression of myosin VIIa in the cytoplasm.

Figure 6. Bone marrow MSCs injected into the otocyst at E3 and harvested at E14

A. An increase in green fluorescence was observed indicating the conversion of bone marrow cells to cells expressing Math1. The cells stained for Math1, myosin VIIa and DAPI (blue). Merge is on the right. B. The green cells were found incorporated into the tissue of the chick otic epithelium. The hair cells of the chick were stained with the chick-specific marker, HCA (white) and myosin VIIa (red), whereas the mouse cells were green due to expression of GFP (arrows). C. Lack of cell fusion was demonstrated by the presence of HCA (arrowhead) in cells that did not have green fluorescence and of *Math1-GFP* (arrow) exclusively in cells that did not stain for HCA, the marker for chicken. No cells with GFP and HCA were observed in these experiments. Scale bars are 100 μ m.

TABLE I
Co-Expression of CD44 and Sca-1 with Nestin in Mesenchymal Stem Cells

Positive cells were counted in relation to total nuclei stained by DAPI. Data are mean $\pm$ SE for 10 separate experiments. The increase in cells staining with nestin was significant ($p < 0.001$) as was the increase in the cells staining for both nestin and CD44 ($p < 0.001$) and nestin and Sca-1 ($p < 0.05$).

	pre-induction (%)	post-induction (%)
Nestin (+) cells	4.7 $\pm$ 0.8	14.2 $\pm$ 2.0
CD44 (+) cells	11.5 $\pm$ 1.6	11.9 $\pm$ 1.8
Sca-1 (+) cells	5.2 $\pm$ 1.5	5.0 $\pm$ 0.4
CD 44 & nestin (+) cells	3.4 $\pm$ 0.9	9.9 $\pm$ 0.9
Sca-1 & nestin (+) cells	2.8 $\pm$ 1.2	4.3 $\pm$ 0.5