Neuronal Differentiation of Bone Marrow-derived Stromal Stem Cells Involves Suppression of Discordant Phenotypes through Gene Silencing*

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Tissue engineering involves the construction of transplantable tissues in which bone marrow aspirates may serve as an accessible source of autogenous multipotential mesenchymal stem cells. Increasing reports indicate that the lineage restriction of adult mesenchymal stem cells may be less established than previously believed, and stem cell-based therapeutics await the establishment of an efficient protocol capable of achieving a prescribed phenotype differentiation. We have investigated how adult mouse bone marrow-derived stromal cells (BMSCs) are guided to neurogenic and osteogenic phenotypes. Naïve BMSCs were found surprisingly active in expression of a wide range of mRNAs and proteins, including those normally reported in terminally differentiated neuronal cells and osteoblasts. The naive BMSCs were found to exhibit voltage-dependent membrane currents similar to the neurally guided BMSCs, although with smaller amplitudes. Once BMSCs were exposed to the osteogenic culture condition, the neuronal characteristics quickly disappeared. Our data suggest that the loss of discordant phenotypes during BMSC differentiation cannot be explained by the selection and elimination of unfit cells from the whole BMSC population. The percent ratio of live to dead BMSCs examined did not change during the first 8–10 days in either neurogenic or osteogenic differentiation media, and cell detachment was estimated at <1%. However, during this period, bone-associated extracellular matrix genes were selectively down-regulated in neurally guided BMSCs. These data indicate that the suppression of discordant phenotypes of differentiating adult stem cells is achieved, at least in part, by silencing of superfluous gene clusters.

Bone marrow represents an abundant source of renewing stem cells with potential for developing into multiple lineages (1–6). Bone marrow transplantations in humans and animals have led to an understanding that the donor’s bone marrow-derived stromal cells (BMSCs) can successfully integrate into a wide range of highly specialized tissues of the recipient in vivo. Transplanted BMSCs have been shown to fuse with differentiated resident cells, such as skeletal and cardiac muscles (7–13), liver (14, 15), and neuronal cells (16–21). However, Houghton et al. (22) have recently shown that the transplanted BMSCs can develop gastric epithelial cells that contain a single nucleus, suggesting that the formation of heterokaryons is not necessarily a prerequisite for BMSC differentiation. It must be noted that their study has further demonstrated that transplanted BMSCs can develop epithelial cancers under chronic Helicobacter infection (22). Regenerative medicine strategy using undifferentiated BMSC transplantation may thus require stringent monitoring.

Bone marrow aspirate can serve as an accessible source of cellular components for tissue engineering strategies. Increasing reports on in vitro differentiation protocols for adult mesenchymal stem cells indicate that the lineage restriction may be less stringent than previously believed. Experiments with multipotent adult stem cells in vitro suggest that the microenvironment contributes substantially to terminal differentiation (12, 14, 23). For example, BMSCs show the potential to adopt widely different end points not only of their well characterized mesenchymal derivatives, such as osteoblasts, chondroblasts, and adipocytes (24–26), but also of ectoderm-derived neural cells (27–33). The in vitro trans-lineage differentiation capability of stem cells should broaden the engineering application to a wide range of tissues, each of which awaits the establishment of an efficient protocol that stably guides them to a prescribed terminal differentiation.

Both rodent and human BMSCs can be rapidly induced to differentiate into neurons in a defined in vitro microenvironment (34). Shortly after the exposure to the neurogenic culture condition, BMSCs begin to develop characteristic neuron-like morphologies, such as processes resembling axons and dendrites (neurites). These cells also express genes and proteins that are normally associated with neuronal cells. We used the adult mouse BMSC model undergoing ectodermal/neurogenic or mesodermal/osteogenic differentiation to elucidate the molecular mechanism regulating the in vitro trans-differentiation

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† The abbreviations used are: BMSC, bone marrow-derived stromal cells; RT, reverse transcription; TTX, tetrodotoxin; TEA, tetraethylammonium chloride; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; ALP, alkaline phosphatase.
Glucose, 25 HEPES, 2 CaCl2, and 2 MgCl2 and was adjusted to pH 7.2. Bone marrow cells were collected from femurs of adult male C57BL/6 mice as described previously (31) and maintained in "control" medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The non-adherent cell population was removed after 48 h, and the adherent BMSC layer was washed twice with fresh medium. The cells were then continuously cultured for 1 week.

For neurogenic induction, subconfluent BMSCs were cultured in the control medium supplemented with 1 μM β-mercaptoethanol (Sigma) for 24 h followed by culture in neurobasal medium supplemented with B27 and 20 ng/ml of brain-derived neurotrophic factor (Invitrogen). For osteogenic induction, subconfluent BMSCs were cultured in neurobasal medium supplemented with 1,8 μM fast red TR (Sigma) and 0.9 μM naphthol AS-MX phosphate (Sigma) in 120 mM Tris buffer (pH 8.4) for 30 min at 37 °C. Nodule mineralization was visualized by treating with 5% silver nitrate solution (Sigma) under ultraviolet light for 30 min, followed by 5% sodium thiosulfate solution (Sigma) for 5 min. The image was scanned, and the area staining for ALP or mineralization was analyzed by Image-Pro Plus version 2.0 (Media Cybernetics, Silver Spring, MD).

### MATERIALS AND METHODS

**BMSC Isolation and Cell Culture**—Mouse BMSCs were obtained by a protocol approved by the UCLA Animal Research Subjects Committee. Bone marrow cells were collected from femurs of adult male C57BL/6 mice as described previously (31) and maintained in "control" medium consisting of minimum essential medium α supplemented with 15% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The non-adherent cell population was removed after 48 h, and the adherent BMSC layer was washed twice with fresh medium. The cells were then continuously cultured for 1 week.

For osteogenic induction, subconfluent BMSCs were cultured in the control medium supplemented with 1% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The non-adherent cell population was removed after 48 h, and the adherent BMSC layer was washed twice with fresh medium. The cells were then continuously cultured for 1 week.

**RT-PCR Analysis**—Total RNA was extracted with TRIzol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). Whole-cell voltage electrophysiology: Patch clamp recordings were made with a patch clamp amplifier (Cairn Optopatch, Cairn Research LTD, Kent, UK) using Clamp software (www.sciifctor.com) and a digital data acquisition board (6052E, National Instruments, Austin, TX). Patch electrodes had a resistance of 3–4 MΩ, and series resistance was below 20 MΩ. The standard internal pipette solution contained (in mM) 10 NaCl, 130 KCl, 1 MgCl2, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, and 0.6 Na-GTP and was adjusted to pH 7.2. The standard external solution contained (in mM) 119 NaCl, 5 KCl, 30 glucose, 25 HEPES, 2 CaCl2, and 2 MgCl2, and was adjusted to pH 7.2. When indicated, tetrodotoxin (TTX, 100 μM) and tetroethylammonium chloride (TEA, 10 μM) were used to block sodium and potassium channels, respectively. Cells were depolarized for 300 ms from a holding potential of −80 mV to the indicated test potentials. Leak subtraction was performed using a standard P/4 protocol (i.e., the hyperpolarizing subtraction pulse was four times smaller than the depolarizing test pulse) and the average current at the end of the depolarization was measured.

**Alkaline Phosphatase (ALP) Assay and von Kossa Staining**—Cells were fixed in 10% buffered formalin phosphate (Fisher) for ALP assay and von Kossa staining. ALP-positive cells were stained by incubating with 1.8 μM fast red TR (Sigma) and 0.9 μM naphthol AS-MX phosphate (Sigma) in 120 mM Tris buffer (pH 8.4) for 30 min at 37 °C. Nodule mineralization was visualized by treating with 5% silver nitrate solution (Sigma) under ultraviolet light for 30 min, followed by 5% sodium thiosulfate solution (Sigma) for 5 min. The image was scanned, and the area staining for ALP or mineralization was analyzed by Image-Pro Plus version 2.0 (Media Cybernetics, Silver Spring, MD).

**Microarray Analysis**—We used our cDNA microarray containing 58 extracellular matrix (ECM) and bone-related genes and 96 internal control genes (35, 36) as follows: bone matrix proteins (osteopontin (3), osteonectin (2), osteocalcin (1), bone sialoprotein); receptors (integrins α2, B1, and B3, vitamin D receptor, parathyroid hormone receptor, estrogen receptor, FGF receptors 2, 3, and 4); osteoblastic markers (ALP, Cbfa1); adhesive proteins (fibronectin, chondroitin sulfate proteoglycan 1, decorin, tenascin X, and syndecan 2); metalloproteinases (matrix metalloproteinase 1 and 2); growth factors (Bmp 2, 4, and 7, TGF-B1,-B2, and -B3, and FGF 2); fibrillar collagens (collagens 1A1, 1A2, 2A1, 3A1, 5A1, 5A2, 11A1 (2), and 11A2); fibril-associated collagen with inter-
ruptured triple helices (FACITs) (collagens 9A1, 9A2, 9A3, 12A1, 16A1, and 19A1); other collagens (collagens 4A1, 4A2, 6A1, 6A3, 7A1, 10A1, and 15A1); and others (Twist, apoE, prolyl 4-hydroxylase, and tissue implant integration-specific gene 1). Briefly, 10 μg of total RNA extracted from osteogenically, neurogenically, and non-induced BMSCs was labeled by incorporating Cy3 or Cy5 during reverse transcription. Labeled probes were mixed, concentrated, and suspended into 10 μg of hybridization solution containing 3 M SSC, 0.2% SDS, 5 M Denhardt’s solution, 3.2 μg of oligo(dA), and 0.4 μg of Cot-1 DNA. After overnight hybridization at 65 °C, microarray slides were scanned on a 418 array scanner (Affymetrix, Inc., Santa Clara, CA), and images were analyzed with ScanAlyze version 2.5 (rana.lbl.gov). Spots with signal intensities <3-fold of background intensity were eliminated. Log2 of the Cy3/Cy5 signal ratio and the summed Cy3+Cy5 intensity were used to qualify changes in gene expression.

RESULTS

Mouse BMSCs maintained in non-inducing medium (control medium) exhibited three distinct morphologies: large flat cells (arrowheads), spindle-shaped cells (thick arrows), and small round cells (thin arrows). Day 1, BMSCs in neurogenic medium (Neu) retract to show processes (arrows) and round cell bodies (arrowheads). Day 2, arrowheads indicate emergence of a neurite-like process with growth cone-like structures. Day 10, arrows indicate an axon terminal-like appearance. Scale bars, 50 μm. B, RT-PCR analysis of neurogenically induced BMSCs expressing the mRNA species encoding β-tubulin III (an early neuronal marker) and nestin (a neuronal stem cell marker), whereas little expression of the neuroectodermal marker Pax6 and the glial markers GFAP and MBP was observed. RNA from mouse whole brain is shown as a positive control. C, amplitudes of membrane currents of neurogenically induced BMSCs evoked by depolarization to the indicated potentials in the presence and absence of TTX and TEA. Inset, representative current traces for BMSC in neurogenic medium (Neu). Neurogenic induction began when BMSCs reached subconfluence (Day 0). Data are mean values ± S.E. with the indicated number of cells for each condition.

Fig. 1. Cellular properties of neurogenically induced BMSCs. A, Day 0, BMSCs in control medium (Con) display three distinct morphologies: large flat cells (arrowheads), spindle-shaped cells (thick arrows), and small round cells (thin arrows). Day 1, BMSCs in neurogenic medium (Neu) retract to show processes (arrows) and round cell bodies (arrowheads). Day 2, arrowheads indicate emergence of a neurite-like process with growth cone-like structures. Scale bars, 50 μm. B, RT-PCR analysis of neurogenically induced BMSCs expressing the mRNA species encoding β-tubulin III (an early neuronal marker) and nestin (a neuronal stem cell marker), whereas little expression of the neuroectodermal marker Pax6 and the glial markers GFAP and MBP was observed. RNA from mouse whole brain is shown as a positive control. C, amplitudes of membrane currents of neurogenically induced BMSCs evoked by depolarization to the indicated potentials in the presence and absence of TTX and TEA. Inset, representative current traces for BMSC in neurogenic medium (Neu). Neurogenic induction began when BMSCs reached subconfluence (Day 0). Data are mean values ± S.E. with the indicated number of cells for each condition.
tin; however, little expression of Pax6, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP) was observed (Fig. 1B). The membrane currents of neurogenically induced BMSCs on day 4 displayed distinct voltage dependence with a peak inward current at a depolarized voltage of −40 mV. TTX eliminated the inward current and unmasked an outward current. The combined application of TEA and TTX almost completely eliminated the inward current and unmasked an outward current.

In osteogenic induction medium, BMSCs exhibited predominantly large, flat cell shapes on day 8, whereas BMSCs in the control medium remained in the original cell shapes (Fig. 2A). The strong expression of osteocalcin, osteopontin, and ALP was observed by day 8 (Fig. 2, B and C), and mineralized nodules were formed covering 19.4% of the culture area by day 15 (Fig. 1B).

While establishing these neurogenic and osteogenic differentiation protocols, we have repeatedly observed that naïve BMSCs cultured in a control medium expressed genes indicative of both neuronal (β-tubulin III and nestin; Fig. 1B) and osteoblastic (osteocalcin, osteopontin, and ALP; Fig. 2B) phenotypes. In addition, the majority of these naïve, uninduced BMSCs also expressed the neuronal proteins Trk A and β-tubulin III. However, their spindle-shaped cell bodies did not display neuronal (neurites) or osteoblastic (mineralized nodules) characteristics (Fig. 3). Staining intensity for neuronal markers in naïve BMSCs was equivalent to that of neurogenically induced BMSCs, which developed distinct, neurite-like processes (Fig. 3, C and G), but no neuronal markers were detected in osteogenically induced BMSCs (Fig. 3, D and H). We further examined the electrophysiological properties of control, neurogenically, and osteogenically induced BMSCs using whole-cell voltage clamp recordings. We were unable to record from osteogenic cells in osteogenically induced BMSCs on day 4 displayed distinct voltage dependence. The expression of voltage-gated sodium and potassium channels, eliminated the inward current and unmasked an outward current. The combined application of the potassium channel blocker TEA and TTX almost completely eliminated all voltage-dependent currents. This suggests that the neurogenically induced BMSCs express functional sodium and potassium channels.

Our experiments showed that the same batch of adult mouse BMSCs can develop ectodermal/neuronal and mesenchymal/osteoblastic phenotypes. The differentiation process of various cellular phenotypes was monitored by observing cell morphology and the expression of mRNA, proteins, and functional cellular properties characteristic of the guided differentiated lineages. We demonstrated that neuronal cells derived from BMSCs expressed mRNAs encoding β-tubulin III (an early neuronal marker) and nestin (a neuronal stem cell marker) (28, 31, 36, 38, 39). However, little expression of the neuroectodermal marker Pax6 and the glial markers GFAP and MBP was observed (Fig. 1B). These BMSCs may differentiate directly to neurons without typical neuroectodermal passages under this culture condition. The neurogenically induced BMSCs on day 4 displayed distinct voltage-dependent membrane currents with a peak inward current at a depolarized voltage of −40 mV. TTX, a specific blocker of voltage-gated sodium channels, eliminated the inward current and unmasked an outward current. The combined application of the potassium channel blocker TEA and TTX almost completely eliminated all voltage-dependent currents. This suggests that the neurogenically induced BMSCs express functional sodium and potassium channels.

**DISCUSSION**

Our experiments showed that the same batch of adult mouse BMSCs can develop ectodermal/neuronal and mesenchymal/osteoblastic phenotypes. The differentiation process of various cellular phenotypes was monitored by observing cell morphology and the expression of mRNA, proteins, and functional cellular properties characteristic of the guided differentiated lineages. We demonstrated that neuronal cells derived from BMSCs expressed mRNAs encoding β-tubulin III (an early neuronal marker) and nestin (a neuronal stem cell marker) (28, 31, 36, 38, 39). However, little expression of the neuroectodermal marker Pax6 and the glial markers GFAP and MBP was observed (Fig. 1B). These BMSCs may differentiate directly to neurons without typical neuroectodermal passages under this culture condition. The neurogenically induced BMSCs on day 4 displayed distinct voltage-dependent membrane currents with a peak inward current at a depolarized voltage of −40 mV. TTX, a specific blocker of voltage-gated sodium channels, eliminated the inward current and unmasked an outward current. The combined application of the potassium channel blocker TEA and TTX almost completely eliminated all voltage-dependent currents. This suggests that the neurogenically induced BMSCs express functional sodium and potassium channels.
neuronal markers, such as nestin, TrkA, and β-tubulin III and the concurrent demonstration of voltage-gated currents are a hallmark of neurogenic differentiation (37, 38, 40, 41) in embryonic and neural stem cells. In the literature, the differentiation process of BMSCs has been followed by the monitoring expression of mRNA, proteins, and functional cellular properties characteristic of the guided differentiated lineages (25, 29, 42). These studies have followed the time-dependent expression profile of these differentiation markers above baseline levels. Our results suggest, however, that the expression of osteogenic and neuronal molecular markers as well as the voltage-dependent currents are a constitutive cellular property of uninduced, naive BMSCs in the absence of differentiation-inducing stimuli (Fig. 3). A recent paper by Tondreau et al. (43) suggested that, after several passages, undifferentiated BMSCs expressed neuronal genes and proteins examined by RT-PCR and Western blot. Taken together, these findings indicate that nonspecific gene expression can be an intrinsic property of adult stem cells.

Isolated BMSCs have been observed in various morphologies representing the heterogeneous nature of BMSCs composed of mesenchymal precursors, including the recently described recycling/rapidly self-renewing stem cells (44, 45) and hematopoietic precursors (46). It must be noted that different cell shapes have also been reported in human clonal BMSCs (44, 45, 47), although the broad and flattened shape eventually predominates after repeated cell divisions (48–51). In the BMSC population, some stem cells may be true pluripotential cells, i.e. multipotential adult progenitor cells (39), whereas others may have already undergone lineage-specific differentiation at various stages. In our study, the BMSCs were not initially screened and contained a mixture of cells ranging from multipotential adult progenitor cells to mesenchymal stem cells with various differentiation commitments. The expression of a mixture of lineage-specific phenotypes by the BMSCs may well represent the collective phenotypic profiles of these heterogenic cells. It has been postulated that hematopoietic cell differentiation undergoes positive and negative selection among various cell populations (52–54). It seemed possible that the down-regulation of unrelated phenotype markers and cellular properties resulted from negative selection. If this were the case, the in vitro differentiation of BMSCs should, in part, employ a selection and elimination process in which unfit cells undergo apoptosis. We addressed this possibility by a simple cell live/dead assay (Fig. 4). To our surprise, cell vitality was well maintained until the second week in both osteogenic and neurogenic differentiation media. A sudden decrease of cell vitality occurred at days 8 and 10 in neurogenic and osteogenic culture condition, respectively. Therefore, negative selection may occur in the late differentiation stages. However, during the early differentiation stages, the negative selection of unfit cells alone cannot explain the down-regulation of unrelated cellular properties. Moreover, during the early differentiation stages, <1% of cells were found detached and floating in the differentiation medium. Most of these detached cells underwent apoptosis. However, it was not determined whether
apoptotic cells exfoliated from the culture dish or the loss of integrin-mediated anchorage due to mechanical dislodgement induced apoptosis, described as anoikis (55). Nonetheless, it is unlikely that selective loss of subpopulations of BMSCs through detachment contributed to the loss of marker gene expression.

It is possible then, that the whole BMSC differentiation process entails a conversion from broad to phenotype-specific gene expression. We showed that such conversion may involve suppression of non-phenotype-specific genes (Fig. 4). During the embryogenesis, the ultimate totipotent cells gave rise to all cell types. Animal development can therefore be described as a progressive loss of totipotency, and then pluripotency, and ultimately differentiation into specific cell types. Using large scale microarray analysis, Tanaka et al. (56) suggest that totipotency of embryonic stem cells requires a set of genes not expressed in other cell types, whereas lineage-restricted stem cells, such as extra-embryonic-restricted trophoblast stem cells, express genes predictive of their differentiated lineage. Adult BMSCs are not totipotent stem cells but are considered lineage-restricted multipotent stem cells, although the phenotype restriction may not be as narrow as previously thought.

The mechanism that regulates adult stem cell differentiation, by pruning a repertoire of differentiation-unrelated genes down to lineage-specific characteristics, may share the underlying mechanism of positive and negative gene regulation during development (57). However, the rapid loss of the steady state level of gene expression activity observed in this study may present a unique property of adult stem cell undergoing phenotype differentiation in the in vitro environment. For tissue engineering applications, it is important to lead stem cells to the targeted end point, which may be guided not only by up-regulating tissue phenotype-sensitive genes but also by suppressing discordant gene expression. We speculate that this transcriptional down-regulation could involve suppressor-like nuclear factors, rapid DNA methylation, or RNA interference. In any event, the postulated “gene pruning” differentiation mechanism may provide the basis for stem cell-based biotechnology and for potential applications in tissue engineering.

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