MULTIPLE DIFFERENTIATION POTENTIALS OF NEONATAL DURA MATER-DERIVED CELLS

Ioana A. Peptan, D.D.S., M.S.
Department of Bioengineering, University of Illinois at Chicago, Chicago, Illinois

Liu Hong, M.D., Ph.D.
Departments of Orthodontics and Bioengineering, University of Illinois at Chicago, Chicago, Illinois

Carla A. Evans, D.D.S., D.M.Sc.
Department of Orthodontics, University of Illinois at Chicago, Chicago, Illinois

Reprint requests:
Liu Hong, M.D., Ph.D., Department of Orthodontics, Stem Cell and Craniofacial Tissue Engineering Laboratory, University of Illinois at Chicago, 801 South Paulina Street, MC 841, Chicago, IL 60612-7211.
Email: liuhong1@uic.edu

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OBJECTIVE: The involvement of the dura mater in calvarial development and bone healing lead to a hypothesis that progenitor cells with multiple differentiation potentials exist within this tissue. The present study investigated the differentiation potentials of dura mater-derived cells by driving them into several cell-restricted lineages.

METHODS: Dissected dura mater tissue of neonatal rats was washed, finely minced, and enzymatically digested. The harvested cells were exposed to different differentiation (osteogenic, adipogenic, and chondrogenic) and basic media.

RESULTS: At defined time points, dura mater-derived cells were observed to differentiate into osteoblastic, adipoblastic, and chondroblastic cells, evidenced by specific biochemical staining. In addition, gene expressions of osteogenesis (alkaline phosphatase, osteocalcin, and osteopontin), chondrogenesis (collagen Type II and aggrecan core protein) and adipogenesis (peroxisome proliferator activated receptor γ-2) were up-regulated in the differentiated dura mater-derived cells, confirmed by polymerase chain reaction.

CONCLUSION: Preliminarily, it was concluded that a subpopulation of multiple potential mesenchymal cells exists in neonatal dura mater, which explains the function of the dura mater on neurocranium development and calvarial bone healing.

KEY WORDS: Differentiation potential, Dura mater-derived cell, In vitro, Mesenchymal

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Cranial bone formation occurs either by intramembranous or by endochondral processes involving mesenchymal progenitor cell differentiation, synthesis, and mineralization of the extracellular matrix. Cranial vault formation takes place through intramembranous ossification of the surface mesenchyme surrounding the developing brain, which is presumably achieved by direct transformation of mesenchymal stem cells into osteoblasts. The dura mater interposing between the cerebral hemispheres and calvarium plays an important role in calvarial morphogenesis and bone healing. The functions of the dura mater differ depending on the anatomic sites. For example, cranial suture-associated dura has a ruling function on suture growth and maintenance, whereas squamous dura mater participates in neurocranium development and bone healing. Osteoblasts cocultured with sutural dura can affect osteoblast differentiation, suggesting that the regional dura mater underlying cranial sutures regulates cranial suture fate through paracrine signaling (23). Anomalies in neurocranium formation are often associated with congenital malformations involving central nervous system development (6). Moreover, the osteogenic properties of the dura are thought to contribute to the regenerative capacity of neurocranium bone defects. The capacity for osteoconduction and osteoinduction is unique to immature or young dura mater. Subtotal calveriectomy with preservation of the dura mater in children younger than 2 years of age and immature animals is associated with complete bone regeneration (7, 11). It is assumed that the bone defects are repaired by osteochondroblastic differentiation of the dura cells, by the local action of dura-derived growth factors on mesenchymal cells, or both (16).

The complex role of the dura mater in calvarial bone formation, bone healing, and cranial suture function leads to the assumption of the existence of mesenchymal progenitor cells within this tissue. We hypothesized that a subpopulation of mesenchymal cells within the

dura mater can be differentiated into several cell lineages. To this end, cells isolated from neonatal rat dura were used to identify their various differentiation potentials under specific stimulations.

MATERIALS AND METHODS

Cell Isolation and Culture

All animals used in the present study were under the supervision of the University of Illinois at Chicago's Animal Care Committee. Dura mater cells were isolated from approximately 80 2-day-old, neonatal Sprague-Dawley rats. After sacrifice, the calvarium of each rat was removed, and the squamous dura mater that covers only the endocranial surface of the calvarium was collected. All harvesting tissue procedures were performed under the dissecting microscope. Squamous dura mater tissue from all rats was washed three times in phosphate-buffered saline (PBS) to remove contaminated tissues and blood. Finely minced tissues were digested with 0.075 wt% collagenase Type I (Worthington, Lakewood, NJ) for 45 minutes at 37°C, with intermittent shaking. The concentration of collagenase used to collect adipose-derived cells was duplicated from previous studies (25). The digested tissue solution was then neutralized and filtered through a cell strainer with a pore size of 70 μm to remove undigested tissues. The collected cells were cultured in basic cell culture medium consisting of Dulbecco's Modified Eagle's Medium (GibcoBRL, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic in a 37°C incubator with 5% CO_2. Culture medium was exchanged every third day. After reaching 70 to 80% confluence, cells were dissociated with 0.25 wt% trypsin/ethylenediamine tetra-acetic acid (GibcoBRL) and subcultured for cell differentiation studies.

Osteogenesis

First-passage dura mater-derived cells (DMDCs) were subcultured in six-well plates at a density of 5×10^5 cells/well and exposed to osteogenic differentiation medium for up to 3 weeks. Osteogenic differentiation medium consisted of basic medium supplemented with 100 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 0.05 mmol/L ascorbic acid-2-phosphate, which has been used extensively to differentiate mesenchymal stem cells into osteoblasts (2, 18). Cells cultured in basic medium served as controls. At each defined time point, cultured cells were collected and subjected to analysis of cell proliferation and osteogenic differentiation. Osteogenic gene expressions were identified by polymerase chain reaction (PCR) analysis.

Deoxyribonucleic Acid Measurement

On Days 7, 14, and 21, cells cultured in six-well plates from both osteoinductive and control groups were washed twice with PBS and then suspended and lysed in 1% Triton-X100 solution (Sigma Chemical Co., St. Louis, MO). The collected cells subsequently were homogenized using sonication (Dismembrator Model 100; Fisher Scientific, Pittsburgh, PA). The deoxyribonucleic acid (DNA) content in the cells was determined by a fluorometric assay using a spectrofluorometer and a DNA quantification kit (Hoechst 33258; BioRad, Hercules, CA). The fluorescent optical density of each sample was measured at an excitation wavelength of 360 nmol/L and an emission wavelength of 460 nmol/L. The amount of DNA in each sample was determined by using a prepared standard curve (19). DNA results were expressed as micrograms per milliliter.

Assessment of Alkaline Phosphatase Activity and Mineralization

The cultured cells were fixed in 10% paraformaldehyde and incubated with 120 mmol/L Tris buffer (pH 8.4) containing 0.9 mmol/L Naphtol AS-MX Phosphate (Sigma Chemical Co.) and 1.8 mmol/L Fast Red TR (Sigma Chemical Co.). Naphtol AS-MX Phosphate was solubilized with *N*,*N*-dimethylformamide (Fisher Scientific) before dilution with Tris buffer. After 45 minutes at room temperature, the cultures were washed with deionized water. Mineral deposition of extracellular matrix was reflected by the presence of black nodules using von Kossa

TABLE 1. Reverse transcriptase polymerase chain reaction primers for osteogenic, adipogenic, and chondrogenic differentiation markers

Gene	Sequence
Osteopontin (*OP*)	Sense: CCTCCTGTCTCCCGGTGAAA
	Antisense: AAACTCGTGGCTCTGATGTT
Osteocalcin (*OC*)	Sense: AGGACCCTCTCTCTGCTCAC
	Antisense: AACGGTGGTGCCATAGATGC
Alkaline phosphatase (*ALP*)	Sense: TCCATGGTGGATTATGCTCA
	Antisense: TTCTGTTCCTGCTCGAGGTT
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	Sense: TGAACGGGAAGCTCACTGG
	Antisense: TCCACCACCCTGTTGCTGTA
Peroxisome proliferator activated receptor γ-2 (*PPAR γ-2*)	Sense: AAACTCTGGGAGATCCTCCT
	Antisense: TCTTGTGAACGGGATGTCTT
Aggregan core protein	Sense: AGGATGGCTTCCACCAGTGT
	Antisense: CATAAAAGACCTCACCCTCC
Collagen Type II	Sense: CTCAAGTCGCTGAACAACC
	Antisense: CTATGTCCACACCAAATTCC

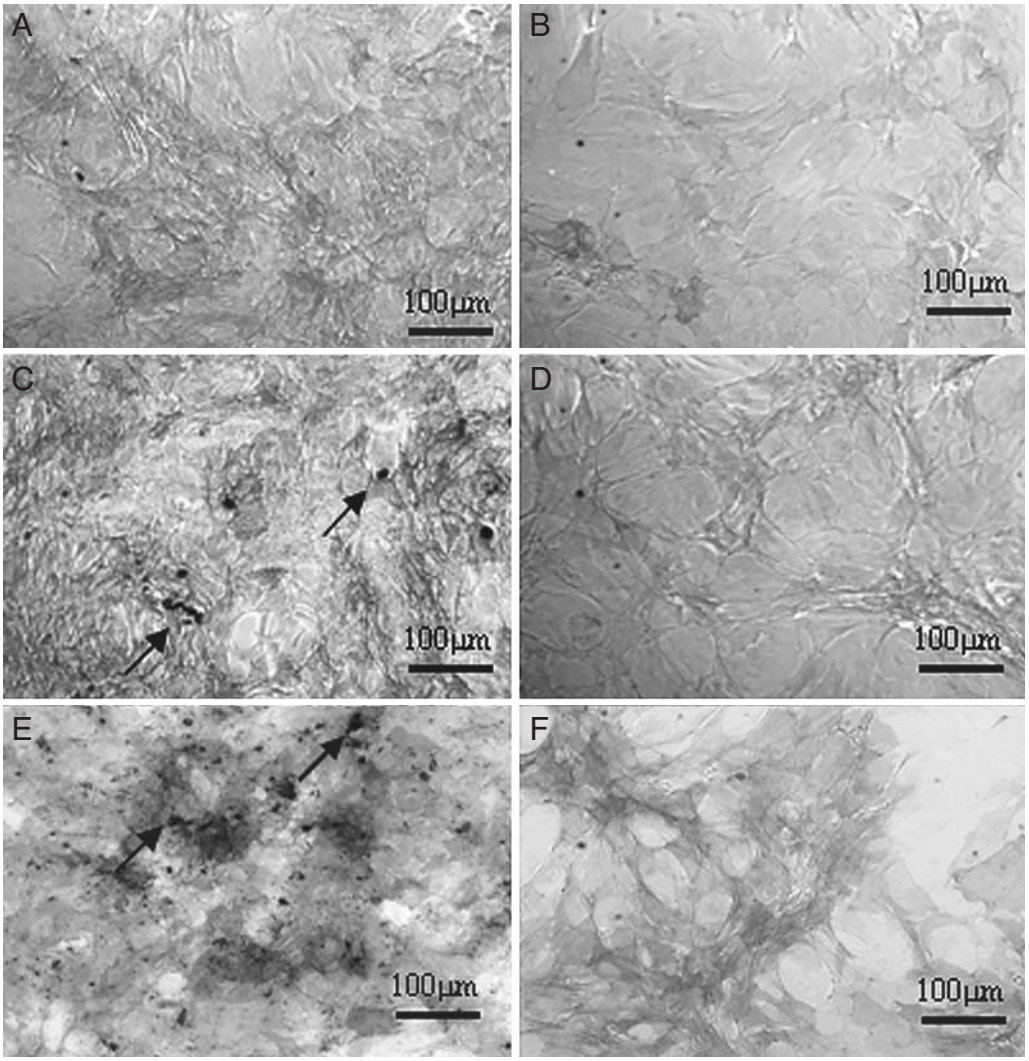

FIGURE 1. *Osteogenesis of fibroblast-like cells isolated from dura mater tissue. Under osteogenic stimulation, dura mater cells showed intense positive reaction to ALP and von Kossa staining at 1 (*A*), 2 (*B*), and 3 (*C, arrows*) weeks. The control group cells exposed to basic medium showed a mild positive reaction to ALP and a negative reaction to von Kossa staining at 1 (*D*), 2 (*E, arrows*), and 3 (*F*) weeks. Original magnification, ×10.*

staining. The fixed cells were incubated in 2.5% (w/v) silver nitrate (Sigma Chemical Co.) solution for 30 minutes in sunlight. The excess silver nitrate was washed away with deionized water. To quantify alkaline phosphatase (ALP) activity and calcium content, cultured cells were washed twice with PBS and then suspended and lysed in 0.5 ml of 1% Triton-X100 solution. The collected cells were subsequently homogenized using sonication. ALP and calcium content of solution were measured by spectrophotometry with a colorimetric kit, according to the manufacturer's instructions (Sigma Chemical Co.).

Adipogenesis

The first passage DMDCs were subcultured at a density of 20,000 cells/cm^2 in adipogenic medium that consists of basic medium supplemented with 50 nmol/L of dexamethasone, 10 mmol/L of insulin, and 5 mmol/L of isobutyl-methylxanthine for 1 week (8). The medium was exchanged three times a week. After 1 week, the adipogenic differentiated cells and control group cells were evaluated by biochemical and molecular analysis.

Oil-O-Red Staining

The cells were fixed in 10% paraformaldehyde at room temperature. Then, the cells were incubated in 2% (w/v) Oil-O-Red reagent (Sigma Chemical Co., St. Louis, MO) for 7 minutes. The excess stain was removed with deionized water followed by counterstaining with hematoxylin. Intracellular lipid accumulation, a marker of adipogenesis, stained in red. Gene expression of peroxisome proliferator-activated receptor (PPAR) γ-2 was investigated to further support adipogenesis.

Chondrogenesis

Chondrogenic differentiation was induced using the high-density "micromass" culture technique. Ten-μL drops of cellular suspension (5×10^7 cells/ml) were placed in petri dishes and incubated for 2 hours at 37°C and 5% CO_2. The dishes were then filled with basic medium and incubated overnight. The next day, chondrogenic supplements consisting of 10 ng/ml transforming growth factor-β1, 6.25 μg/ml insulin, 6.25 μg/ml transferrine, and 0.1 μmol/L dexamethasone were added to basic medium (4, 21). On defined days, cell masses were collected for histological evaluation. Gene expressions of collagen Type II and aggregan core protein were used to evaluate chondrogenesis of DMDCs. Total ribonucleic acid (RNA) was extracted from cells subcultured for 3 weeks in chondrogenic and basic medium using TRIzol reagent (GibcoBRL). PCR was used to identify chondrogenic gene expression.

Alcian Blue Staining

Alcian blue staining is specific for visualizing sulfated proteoglycans during chondrogenesis (16). Before staining, the cellular masses were washed twice with PBS and fixed in 10%

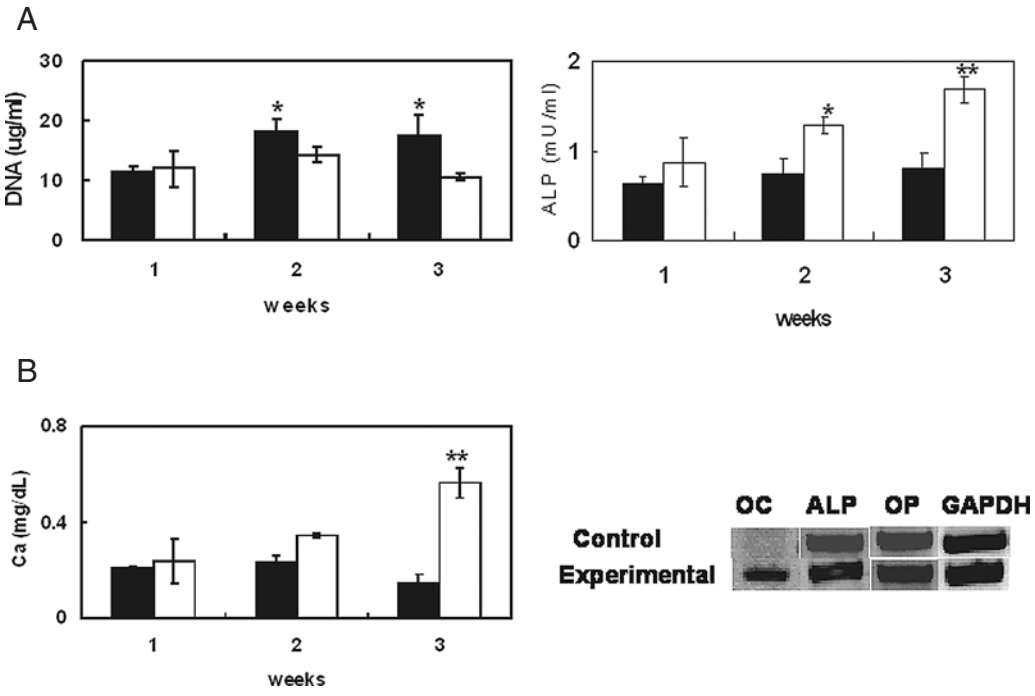

FIGURE 2. *Quantitative assessment of osteogenic differentiation potential. The proliferation rate of DMDCs exposed to osteogenic medium decreased with time compared with that of the control group (*A*). In the osteogenic differentiation group, ALP activity and calcium content (*Ca*) increased during a 3-week period (*B*) and were significantly greater in the osteogenic differentiation group than the control group (*B, C*). PCR detection of messenger RNA for osteocalcin (*OC*), ALP, osteopontin (*OP*), and GAPDH was performed after 3 weeks of differentiation. The PCR products were analyzed on an ethidium bromide-stained agarose gel (*D*).* Asterisk, $P < 0.05$; double asterisk, $P < 0.01$.

paraformaldehyde. The samples were embedded in paraffin and cut into 5-μm sections. The slides were incubated for 30 minutes with 1% (w/v) Alcian blue (Sigma Chemical Co.) in 0.1 N HCl (pH 1.0) and washed with 0.1 N HCl for 5 minutes to remove excess stain.

PCR Analyses

Total RNA was extracted from subcultured cells using TRIzol reagent according to the manufacturer's protocol. Approximately 5 μg of total RNA was converted to single-stranded complementary DNA (cDNA) using a commercial cDNA synthesis kit (GIBCO Life Sciences). Aliquots of the cDNA were amplified with Ampli-*Taq* DNA polymerase (PerkinElmer, Norwalk, CT) for ALP, osteocalcin, osteopontin, collagen Type II, aggrecan core protein, and *PPAR*γ-2 genes. Thirty-five cycles were used for all genes, each consisting of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 60°C, and 1 minute of polymerization at 72°C, followed by a final 10 minutes extension at 72°C. The housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as a control for RNA loading samples. PCR products were analyzed electrophoretically with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) on 2.0% agarose gel and visualized by staining with ethidium bromide. The primer sequences used in this study for PCR analysis are summarized in *Table 1*.

Statistical Analysis

All quantitative data were expressed as mean ± standard deviation. Student's *t* tests were used to compare the extent of osteogenic differentiation between treatment and control groups. *P* values less than 0.05 were considered significant.

RESULTS

Osteogenic Potentials

Fibroblast-like cells were isolated from neonatal rat dura mater tissue. DMDCs were proliferated by in vitro culture in basic medium. After exposure to osteogenic differentiation medium, DMDCs formed an extensive network of dense, multilayered nodules with high ALP activities and mineral deposition. ALP activity and mineralization content increased as experimental times were extended (*Fig. 1, A–C*). In contrast, the control group cells showed mild positive reaction to ALP staining but no mineralization (*Fig. 1, D–F*). Quantitative assessment of cell proliferation revealed that the proliferation rate of DMDCs slowed after exposure to osteogenic differentiation medium, as compared with control cells exposed to basic medium (*Fig. 2A*). ALP activity and calcium content of DMDCs exposed to osteogenic differentiation increased with time and were significantly higher than controls (*Fig. 2, B* and *C*). Osteogenic gene expression revealed that, although ALP and osteopontin were expressed in control and treatment groups, osteocalcin was only expressed in the osteogenic differentiation group (*Fig. 2D*).

Adipogenic Potentials

After 1 week of incubation with adipogenic differentiation medium, a few cells morphologically resembled adipocytes, exhibiting a rounded appearance. Under a higher magnification of inverted microscopy, intracellular lipid vesicles were observed (*Fig. 3A*). A positive reaction of Oil-O-Red staining demonstrated accumulations of lipid droplets in monolayer-cultured DMDCs under adipogenic differentiation (*Fig. 3B*). In contrast, control cells exposed to the nondifferentiation medium retained their fibroblast-like spindle shapes, and no positive reaction to Oil-O-Red staining was found (*Fig. 3C*). *PPAR*γ-2 gene, a specific adipogenic marker, was expressed at 1 week of adipogenic differentiation in the treatment group. No band was observed in the control group exposed to basic medium (*Fig. 3D*).

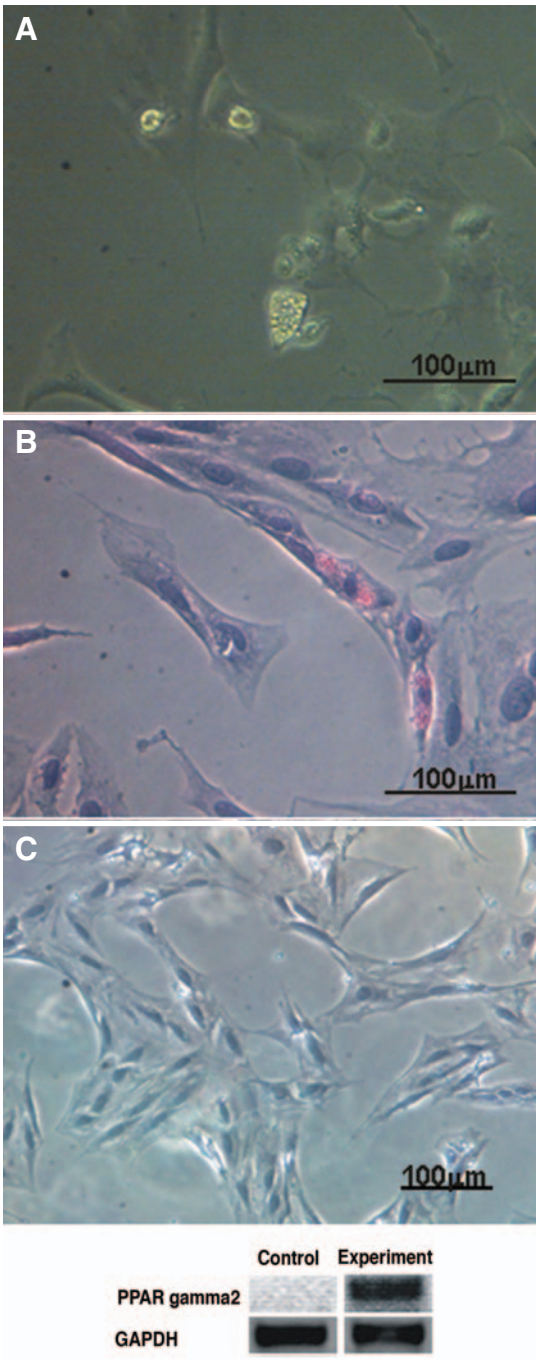

FIGURE 3. *DMDCs exposed to adipogenic medium for 1 week demonstrated intracellular lipid accumulation* (A)*, which stained positively with Oil-O-Red* (B)*. Cells cultured in basic medium lacked intracellular lipid accumulation* (C)*. Original magnification,* ×*40. PCR detection of mRNAs for* PPARγ*-2 and* GAPDH *was performed after 1 week of differentiation. The PCR products were analyzed on an ethidium bromide-stained agarose gel* (D)*.*

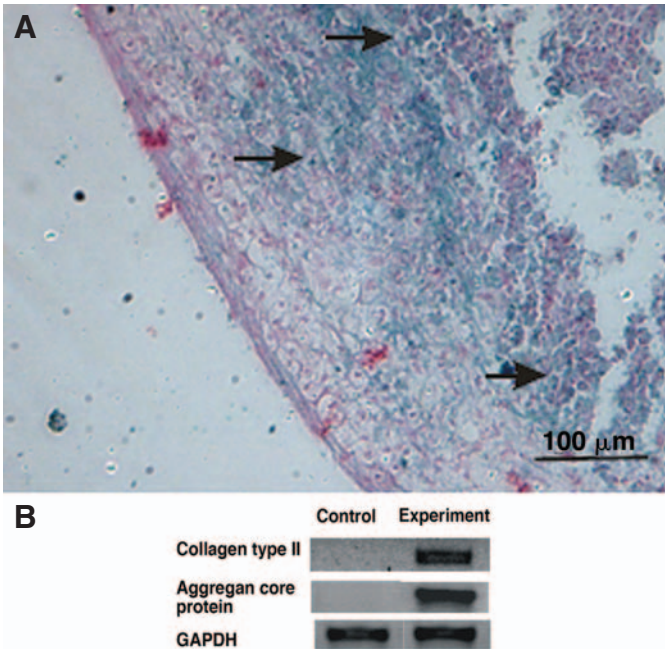

FIGURE 4. *Under chondrogenic stimulation, DMDCs formed spherical pellets that stained positively with Alcian blue staining* (A, arrows)*. PCR detection of mRNAs for collagen Type II, aggregan core protein, and* GAPDH *was performed after 3 weeks of differentiation. The PCR products were analyzed on an ethidium bromide-stained agarose gel* (B)*.*

Chondrogenic Potentials

DMDCs cultured under micromass conditions formed cell pellets associated with a well-organized extracellular matrix rich in sulfated proteoglycans and collagen Type II. Up to 3 weeks, the DMDCs pellets were positive for Alcian blue staining, indicating the presence of sulfated proteoglycans (*Fig. 4A*). Collagen Type II and aggregan core protein, genes of specific chondrogenic markers, were up-regulated after 3 weeks of differentiation (*Fig. 4B*). The control group failed to express any chondrogenic markers (*Fig. 4B*).

DISCUSSION

Cell lineage studies using embryo cultures or short-term labeling techniques (β-galactosidase, dioctadecylindocarbocyanine, and X-gal staining) elucidated neural crest cell migration pathways (10, 17, 20). These studies demonstrated that meninges (including dura mater) originate from neural crest cells. During craniofacial development, neural crest cells migrate ventrolaterally as they populate the branchial arches. These ectodermally derived cells are multipotent stem cells that contribute significantly to the formation of mesenchymal structures in the head and neck (1, 14). In this study, we examined the multilineage potential of a putative ectodermal cell population obtained from rat neonatal dura mater. After dissimilar differentiation stimulations, osteogenic, adipogenic, and chondrogenic potentials of the cells were demonstrated by his-

tological and biochemical techniques. Although the capacity of dura mater tissue to form bone and cartilage ectopically was previously reported (24), this study is the first one to our knowledge to identify mesenchymal cells with multiple differentiation potentials within neonatal squamous dura mater. It preliminarily explains the function of the dura mater on neurocranium development and calvarial bone healing.

The dura mater has been demonstrated to play a critical role in calvarial morphogenesis. Early embryonic skeletogenesis requires intimate interactions between surface epithelium and the dura mater. Mehrara et al. (12) showed that the dura mater underlying the developing calvarial bone strongly expressed transforming growth factor-β1 and fibroblast growth factor-2 messenger RNA (mRNA) from embryonic until neonatal age, and that was attributed to cranial vault bone formation. Moreover, they found that calvarial osteoblasts located near the endocranial surface and in contact with the developing dura were strongly stained for transforming growth factor-β1 and fibroblast growth factor-2. The dura mater has also been shown to manipulate calvarial bone healing. Uddstromer and Ritsila (22) showed that neonatal dura posses a stronger osteogenic capacity than periosteum during the healing of membraneous cranial bone defects. Warren et al. (23) demonstrated that the underlying dura of coronal and sagittal sutures can affect osteoblast differentiation, indicating that regional dura mater regulates the fate of cranial sutures through paracrine signaling. In addition, Yu et al. (24) found that different regional dura have different functions and capabilities of differentiation, indicating that squamous dura might contain different cell phenotypes from sutural dura. This suggests that the dura mater of young animals can turn into bone and cartilage formation when transplanted in epithelial pouches. The present study demonstrates that DMDCs possess multiple differentiation potentials under specific differentiation conditions, which are similar to those of mesenchymal stem cells.

The existence of mesenchymal stem cells with multiple differentiation potentials has been demonstrated in bone marrow, trabecular bone, adipose tissue, synovium, skeletal muscle, and dental pulp (3, 5, 9, 13, 15, 25). They act as reservoirs of reparative cells, ready to differentiate and mobilize in response to growth and wound signals or pathological conditions. However, the population of these progenitor cells reduces with age. Similarly, adult DMDCs were found in a parallel study of our laboratory to lose the characteristics of multiple differentiation capabilities but maintain their osteogenic potentials (data not shown) There are not available specific markers for the mesenchymal stem cells. The basic criteria for identifying stem cells are multiple differentiation potentials and the ability to self-renew. In the present study, neonatal DMDCs demonstrated osteogenic, chondrogenic, and adipogenic differentiation, as illustrated by the presence of specific cell lineage markers: osteocalcin, collagen Type II, aggregan core protein, and PPARγ-2. The plasticity of the commitment to different cell lineages under specific stimuli is a primordial feature of mesenchymal stem cells. Because of the role of dura in cranial suture development, the preliminary understanding of the mechanism of function of dura cell phenotypes in this study is useful in further investigations for craniosynostosis treatment and craniofacial bone healing. To further confirm DMDC characteristics as those of mesenchymal stem cells, self-renewing capability of the DMDCs and differentiation potentials of DMDCs to other mesodermal lineages (myogenesis) must be identified. Adult dura is demonstrated to have reduced bone formation capabilities as compared with young dura (7). The mechanism might be answered by quantitative comparison studies of multiple potential cell populations among different ages. In addition, the role of these pluripotent cells in neonatal dura need to be further characterized in the future.

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COMMENTS

In this report, the authors provide evidence that neonatal dura mater-derived cells have multiple differentiation potentials. They found that dura mater-derived cells can be driven into specific cell-restricted lineages by exposure to various differentiation media. Although these findings are important and interesting, the implications of the presence of cells with multiple differentiation potential remain unclear. It may turn out that the existence of these cells plays a role in neurocranial development and calvarial bone healing, as the authors suggest. However, there is no conclusive evidence of this in the present study. Alternatively, these cells may not play any important role. In this study, as with others involving stem cells in other tissue, the gap between existence and functional role can be difficult to bridge. Nevertheless, the results of this study are interesting and the role of dura mater-derived cells deserves further study.

Charles Y. Liu
Los Angeles, California

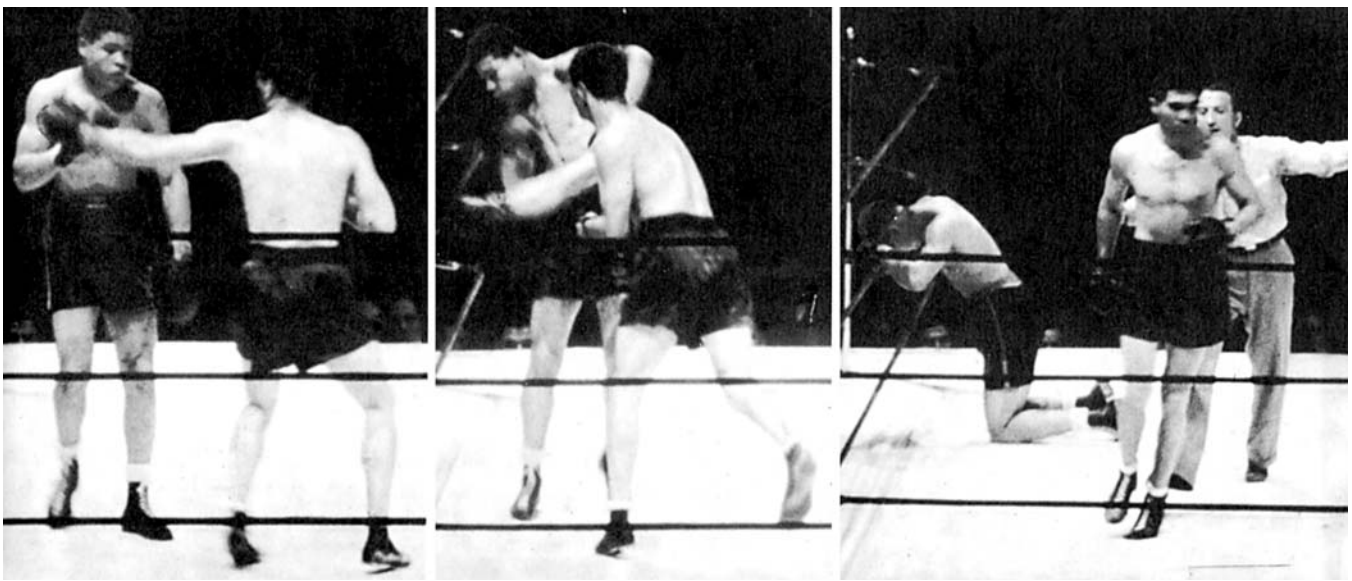
This interesting study provides the reader with a background on dural biology and cranial development. There are some questions related to the authors' methods, including the effects of differentiation agents or collagenase. It is not likely that the dura will prove to be a source for cellular repair. The clinical relevance of the work is somewhat questionable and has potential ramifications for craniosynostosis surgery.

Douglas Kondziolka
Pittsburgh, Pennsylvania

The authors have demonstrated the existence of progenitor cells within the neonatal rat squamous dura mater and have shown their multiple differentiation potential using osteogenic, adipogenic, and chondrogenic stimulation in vitro. Interestingly, similar tissue from adult rats is reported to lose this capacity and maintain only an osteogenic potential.

To further evaluate the properties of these progenitor cells, it will be interesting to learn more about their potential in vivo, e.g., after allogenic transplantation into adequate osteogenic, adipogenic, and chondrogenic niches.

Håvard Ølstørn
Iver A. Langmoen
Oslo, Norway



Historic 12th round knockout sequence from the first Joe Louis (1914–1981) (left)—Max Schmeling (1905–2005) bout, Yankee Stadium, New York, June 19, 1936.