Osteoblastic Differentiation of Wharton Jelly Biopsy Specimens and Their Mesenchymal Stromal Cells after Serum-Free Culture

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Background: Cleft lip and cleft palate are increasingly being detected by prenatal ultrasound, which raises the opportunity of using the patient's own osteogenicity from umbilical cord mesenchymal cells for bony repair. The authors address the growth of the cells under a fully defined and regulated protocol.
Methods: Wharton jelly–derived mesenchymal stromal cells were isolated and expanded as a monolayer with defined serum-free medium. Osteoblastic differentiation was tested in the cells and in the entire Wharton jelly biopsy specimens. The serum-free–cultured cells were included in hydroxyapatite granule–fibrin constructs and, without predifferentiation, subcutaneously implanted into immunoincompetent mice.
Results: Isolation and expansion of Wharton jelly–derived mesenchymal stromal cells were consistently successful under serum-free conditions, and the cells expressed standard mesenchymal stromal cell markers. The serum-free–cultivated cells produced a mineralized extracellular matrix under osteogenic differentiation, with a significant increase of osteoblastic lineage gene expression (*Hox-A10* and *Runx2*) and an up-regulation of downstream osteogenic genes (*OSX*, *OCN*, *ALPL*, and *BSP2*). In vivo, they formed a dense matrix adjacent to the granules after 8 weeks, but no lamellar bone. serum-free–cultivated entire Wharton jelly biopsy specimens produced a mineralized extracellular matrix within the collagen matrix of the Wharton jelly.
Conclusions: The osteogenic differentiation potential of Wharton jelly–derived mesenchymal stromal cells was maintained under serum-free isolation and expansion techniques. The cells without predifferentiation form a dense collagen matrix but not bone in vivo. Moreover, entire Wharton jelly biopsy specimens showed periosteal-like mineralization under osteogenic differentiation, which offers new options for autologous bone tissue engineering, including cleft palate surgery. (*Plast. Reconstr. Surg.* 134: 59e, 2014.)

The stem cell type, its regenerative potential, and the intended therapeutic use must be closely aligned to increase the chance of successful clinical translation. The use of animal-derived products considerably limits the feasibility of developing a validated cell culture protocol to clinical standards.[1,2] We therefore focused on testing the osteogenic potential of

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Wharton jelly-derived mesenchymal stromal cells and Wharton jelly tissue using a serum-free and animal protein-free culture medium (SPE-IV; ABCell-Bio, Paris, France).

In newborns, the umbilical cord is—in contrast to fat or bone marrow—an abundant source of mesenchymal stromal cells without donor-site morbidity. Because the cleft lip and palate are recognized at birth, the patient's own Wharton jelly–derived mesenchymal stromal cells could be preserved for bone regeneration. Stem cell–induced bone regeneration for cleft patients would be interesting beyond its use as an autologous bone graft. More importantly, bone regeneration that complies with craniofacial growth could be targeted.

Reconstruction of the alveolar cleft commonly involves the use of autologous bone from the iliac bone. This method has important shortcomings, including transient donor-site morbidity and the reconstruction of the alveolar graft needing to be delayed to preteen age, because earlier grafting leads to growth inhibition and graft resorption. Thus, an autologous, tissue-engineered material with no donor-site morbidity and that can be used early in life and complies with craniofacial bone growth would provide multiple benefits to the patient.

The challenge of rendering clinically possible the use of Wharton jelly–derived mesenchymal stromal cells for autologous osteogenic tissue engineering for cleft lip–cleft palate was addressed in the present study by evaluating the following three factors:

1. Efficacy to isolate, store and, expand Wharton jelly–derived mesenchymal stromal cells under serum-free conditions and its efficacy for subsequent osteoblastic differentiation.
2. In vivo survival and tissue formation by naive Wharton jelly–derived mesenchymal stromal cells seeded onto three-dimensional constructs, made under serum-free conditions.
3. In vitro osteoblastic differentiation of Wharton jelly biopsy specimens subsequent to cultivation under serum-free conditions.

MATERIALS AND METHODS

serum-free Isolation, Expansion, and Cryopreservation of Wharton Jelly–Derived Mesenchymal Stromal Cells

Human umbilical cord samples for research purpose were taken after written informed consent, using guidelines approved by the institutional review board "comité de protection des personnes Sud-Est, Lyon." Human umbilical cord was stored at room temperature in phosphate-buffered saline with penicillin, streptomycin, and amphotericin B (100 U/ml, 100 μg/ml, and 0.25 μg/ml, respectively) and processed within 48 hours after delivery. Adherent cells were isolated using the explant method.[3] Briefly, 2-mm-thick slices were cut from the cord and 2.5-mm diameter biopsy specimens (MIL3331B; Integra Miltex, Plainsboro, N.J.) were taken from these slices, in between the two umbilical arteries and the vein (Fig. 1). Isolation dishes (35 mm in diameter) were coated with a 4:1 mixture of phosphate-buffered saline and 200 μg/ml collagen I to III from human placenta (ABCell-Bio, Paris, France), replaced with serum-free medium SPE-IV (ABCell-Bio) and four Wharton jelly biopsy specimens, and maintained in a humidified atmosphere with 5% carbon dioxide at 37°C. The medium contained clinical-grade human albumin and recombinant human growth factors. The cells were first passaged to a surface ratio of 1:3 to 1:5 after approximately 2 weeks using a cell-dissociation reagent that is free of animal- and human-derived components (TrypLE Select; Gibco/Invitrogen, Carlsbad, Calif.).

A 5100 Cryo 1°C freezing container was used for cryopreservation (Nalgene, Rochester, N.Y.). Cryopreservation medium that was free of human and animal components (Cryo3, reference no. 5617; Stem Alpha, St. Genis L'Argentière, France) was mixed with 10% dimethyl sulfoxide (reference no. 8418; Sigma-Aldrich, St. Louis, Mo.). Three Wharton jelly biopsy specimens or Wharton jelly–derived mesenchymal stromal cells from a confluent T-25 flask were suspended in 1.8 ml of cryopreservation-dimethylsulfoxide solution. The box was stored at −80°C.

Osteogenic Differentiation Medium

The osteogenic medium contained high-glucose Dulbecco's Modified Eagle Medium (reference no. GIBCO 41966, Invitrogen) supplemented with 10% low–immunoglobulin G fetal bovine serum (reference no. DE14-870E; Lonza, Basel, Switzerland) and the following osteogenic factors: 10 mM β-glycerol phosphate (reference no. G9422; Sigma-Aldrich), 50 μg/ml ascorbic acid (reference no. A4403; Sigma-Aldrich), and 10 nM dexamethasone (reference no. D2915; Sigma-Aldrich). The osteogenic control medium was free of any osteogenic factors. The complete media were stored in the dark at 4°C and used within 1 month. The osteogenic differentiation was initiated at a culture confluence of 80%, and the medium was changed twice per week for 3 weeks.

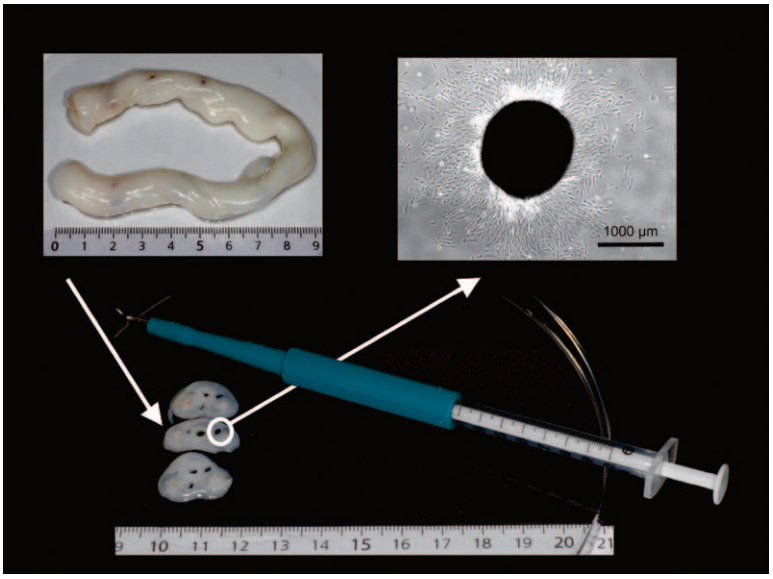

Fig. 1. Isolation of Wharton jelly–derived mesenchymal stromal cells. The umbilical cord (*above*, *left*) is sliced into slices of 2-mm thickness (*below*, *left*). A skin biopsy tool of 2.5-mm diameter is connected to a 1-ml syringe (*below*, *right*) to take Wharton jelly biopsy specimens (*above*, *right*). Cell outgrowth from the Wharton jelly biopsy specimens takes place after 1 week by plastic adherence (increments on the scale are millimeters).

Constructs of Wharton Jelly–Derived Mesenchymal Stromal Cells, Fibrin, and Hydroxyapatite and In Vivo Test

Wharton jelly–derived mesenchymal stromal cells isolated and expanded with serum-free at passages 2 and 3 were combined with fibrin sealant (TISSEEL Kit; Baxter Innovations, Wien, Austria) and porous silicate-substituted hydroxyapatite (Actifuse Microgranules; ApaTech, Hertfordshire, United Kingdom). Wharton jelly–derived mesenchymal stromal cells (3×10^6) were suspended in 30 µl of fibrinogen (40 mg/ml) and mixed with hydroxyapatite microgranules (60 mm^3) that had been washed with Serum-free medium. Coagulation was triggered by adding 30 µl of thrombin (12 IU/ml), and the preparation was incubated for 15 minutes at 37°C and 5% carbon dioxide. Constructs without cells served as controls. For in vivo experiments, the constructs were implanted subcutaneously in the backs of five female, 6-week-old, immunoincompetent mice (CD-Nu/NU; Charles River Laboratories, Wilmington, Mass.). The experiment was conducted in duplicate and the mice were killed 8 weeks after implantation (animal permit no. 1797).

Fluorescence-Activated Cell Sorting

Wharton jelly–derived mesenchymal stromal cells isolated and expanded with serum-free were incubated for 30 minutes at 4°C with fluorochrome-conjugated antibodies (Table 1). The cells were analyzed using fluorescence-activated cell sorting with the aid of a FACSCanto flow cytometer and software, by collecting 10,000 events (BD Biosciences, San Jose, Calif.).

Quantitative Real-Time Polymerase Chain Reaction

Wharton jelly–derived mesenchymal stromal cells isolated and expanded with serum-free were expanded for 3 weeks in osteogenic medium and

Table 1. Antibodies for Fluorescence-Activated Cell Sorting

Marker	Fluorochrome	Provider	Reference No.
CD10	PE	BD Biosciences	555375
CD13	APC	BD Biosciences	557454
CD15	V450	BD Biosciences	642917
CD31	FITC	BD Biosciences	555445
CD34	APC	BD Biosciences	555824
CD44	APC-H7	BD Biosciences	560532
CD45	V500	BD Biosciences	560777
CD73	PE-Cy7	BD Biosciences	561258
CD90	FITC	BD Biosciences	555595
CD105	PE	BD Biosciences	560839
CD133	PE	Miltenyi Biotech*	130-090-853
CD106	FITC	BD Biosciences	551146
CD166	PE	BD Biosciences	559263
HLA-ABC	V450	BD Biosciences	561346
HLA-DR	V500	BD Biosciences	561224

FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.
*Miltenyi Biotech GmbH, Bergisch Gladbach, Germany.

in control medium. Total RNA (4 μg) was used in the reverse transcription to synthesize 4 μg of cDNA (high-capacity cDNA reverse transcription kit; Invitrogen). The reaction was carried out in a thermal cycler TC-512 (Techne, Minneapolis, Minn.). The template was 100 ng of cDNA in the presence of 0.25 μl of forward and reverse gene-specific primers (Table 2), and 10 μl of EXPRESS SYBR GreenER qPCR Supermix Universal (Invitrogen). The amplifications were performed on Mastercycler ep realplex2 (Eppendorf, Hamburg, Germany). The relative gene expression levels were calculated as $2^{-\Delta\Delta^{Ct}}$ using four samples for every *t* test, and three housekeeping genes in replicates.[4]

Staining and Labeling

Samples were fixed with 4% paraformaldehyde (reference no. HT5011; Sigma-Aldrich) for 10 minutes. Alizarin red stain was prepared fresh (20 mg/ml), filtered, and then left for 2 minutes on the cells. The OsteoImage mineralization assay was used for specific green-fluorescence labeling of the hydroxyapatite (reference no. PA-1503; Lonza). Von Kossa staining was performed in 5% silver nitrate under ultraviolet light with 2% sodium thiosulfate, followed by counterstaining with hematoxylin of Mayer.

The implanted constructs were excised as a single piece, fixed in 1% paraformaldehyde, and decalcified in ethylenediaminetetraacetic acid–based decalcification solution for 2 weeks. The decalcified samples were embedded in paraffin, sectioned at 7 μm, and stained with Masson trichrome (reference no. 361350; Réactifs RAL, Martillac, France). Cells of human origin were identified as described previously for human-specific Alu sequences with the aid of a ZytoFast CMV chromogenic in situ hybridization kit (ZytoVision, Bremerhaven, Germany).[5]

Immunohistochemistry

The slides were incubated overnight at 4°C with polyclonal anti-BSP2 antibodies (reference no. ALX-210–312; Enzo, Farmingdale, N.Y.) diluted in phosphate-buffered saline/bovine serum albumin 3% to 1:100. Secondary antibody was applied (reference no. K4002; Dako, Baar, Switzerland) and precipitated by diaminobenzidine (reference no. K3468; Dako). Counterstaining was performed with Mayer hematoxylin.

RESULTS

Isolation, Expansion, and Storage of serum-free Wharton Jelly–Derived Mesenchymal Stromal Cells

Isolation of Wharton jelly–derived mesenchymal stromal cells from biopsy specimens of human umbilical cord always succeeded ($n = 47$) (Fig. 1). Although most of the samples ($n = 31$) were from spontaneous rather than cesarean deliveries ($n = 16$), no bacterial or fungal contamination occurred. After 1 week, the Wharton jelly biopsy specimens had condensed and the emigration of spindle-shaped cells had commenced (Fig. 1, *above, right*). Vials containing Wharton jelly biopsy specimens or Wharton jelly–derived mesenchymal stromal cells at passage 1 and stored at –80°C could always be thawed and reexpanded. The longest period of freezing time was 1 year.

Surface Markers of serum-free–Isolated Wharton Jelly–Derived Mesenchymal Stromal Cells

The surface marker expression of the Wharton jelly–derived mesenchymal stromal cells was analyzed after isolation, cryopreservation for 1 month at passage 1, and reexpansion in serum-free medium to passages 3 to 5. The donors were the same as for the in vivo experiments. Wharton jelly–derived mesenchymal stromal cells expressed standard mesenchymal stromal cell markers but were negative for hematopoietic markers (Fig. 2). Wharton jelly–derived mesenchymal stromal cells expressed CD73, CD44, CD13, CD90, CD10, HLA-ABC, CD105,

Table 2. Primers for Quantitative Real-Time Polymerase Chain Reaction of Gene Expression

Gene	Reference No.	BP	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
ALPL	NM_000478.4	132	TATAAGGCGGCGGGGGTGGT	TGTTCCAATCCTGCGCAGAGCAC
BGLAP (OCN)	NM_199173.4	159	CCTATTGGCCCTGGCCGCAC	ACTGGGGCTCCCAGCCATTGA
HOX-A10	NM_018951.3	167	GAGCGAGCCCTCGATTCGCC	GAATTGCCCAGGGAATCCTTCTCCG
IBSP (BSP2)	NM_004967.3	76	CCAGAGGAAGCAATCACCAAA	TTGAGAAAGCACAGGCCATTC
OSX	NM_001173467.1	77	CACTCTCCCTGCCAGACCTCCAG	GCAGATGGAGAGAGCTGGGGGAAC
RUNX2	NM_001024630.3	128	ACAGAACCACAAGTGCGGTGCA	TGCTTGCAGCCTTAAATGACTCTGT
GAPDH	NM_002046.3	170	GGCTGGGGCTCATTTGCAGGG	TGACCTTGGCCAGGGGTGCT
r18S	NM_022551.2	345	ACCAACATCGATGGGCGGCG	TCGGACACGAAGGCCCCAGA
PTK9	BT019691.1	190	CCAGACCGGCATCCAAGCAAGT	TGGTTGTTTGTCCTCCAACAGGGG

BP, product size in base pairs.

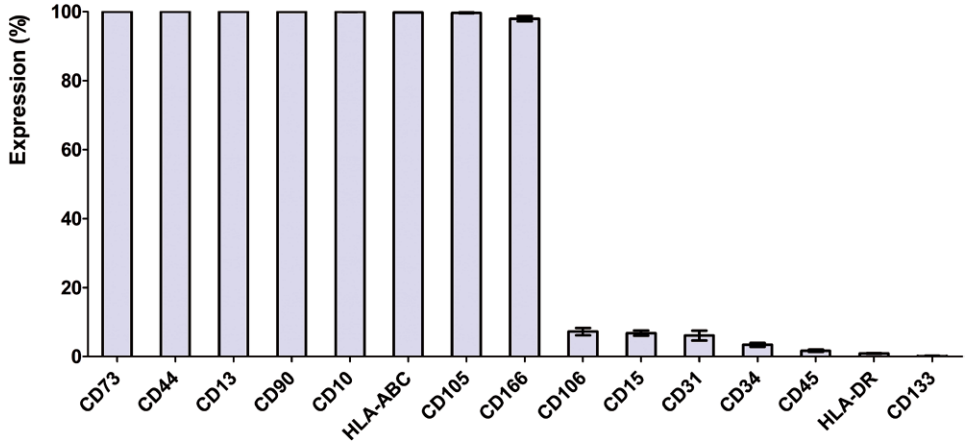

Fig. 2. Mean ± SD values of flow cytometric expression of surface markers in Wharton jelly–derived mesenchymal stromal cells after isolation, cryopreservation, and expansion in serum-free media ($n = 5$; passages, ≤5): CD73, 100 ± 0 percent; CD44, 100 ± 0 percent; CD13, 100 ± 0 percent; CD90, 100 ± 0 percent; CD10, 100 ± 0.1 percent; HLA-ABC, 99.8 ± 0.1 percent; CD105, 99.7 ± 0.3 percent; CD166, 98.0 ± 1.7 percent; CD106, 7.3 ± 2.6 percent; CD15, 6.8 ± 1.9 percent; CD31, 6.1 ± 3.6 percent; C34, 3.4 ± 1.4 percent; CD45, 1.7 ± 0.8 percent; HLA-DR, 0.9 ± 0.3 percent; and CD133, 0.1 ± 0.2 percent.

and CD166 but not (or to a very low extent) CD106, CD15, CD31, CD34, CD45, HLA-DR, and CD133. This spectrum of marker expression is typically known from mesenchymal stromal cells derived from adipose tissue and bone marrow (Table 3).[2,6–16]

In Vitro Monolayer Osteogenic Differentiation of Wharton Jelly–Derived Mesenchymal Stromal Cells

In contrast to the controls (Fig. 3, *below*), the osteodifferentiation samples exhibited mineralization, positive for hydroxyapatite staining with

Table 3. Mesenchymal Stromal Cell Marker Expression in Serum-Free–Cultured Wharton Jelly–Derived Mesenchymal Stromal Cells Compared with Known Expression in BMMSC, ATMSC and Wharton Jelly–Derived Mesenchymal Stromal Cells

	CD 73	CD 44	CD 13	CD 90	CD 10	HLA ABC (MHC-I)	CD 105	CD 166	CD 106	CD 15	CD 31	CD 34	CD 45	HLA-DR (MHC-II)	CD 133
Serum-free WJMSC	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
BMMSC‡	+	+	+	+	+	+	+	+	+	±	–	–	–	–	–
ATMSC†	+	+	+	+	+	+	+	+	±	–	–	±	–	–	–
WJMSC*	+	+	+	+	+	+	+	+	±	NA	–	–	–	–	NA

+, positive expression; _, negative expression; ±, variable expression; NA, not applicable; MSC, mesenchymal stromal cell; WJMSC, Wharton-jelly derived mesenchymal stromal cells; BMMSC, bone marrow–derived mesenchymal stromal cells; ATMSC, adipose tissue–derived mesenchymal stromal cells.
*Results compiled from references 6 through 13.
†Results compiled from references 2 and 14 through 16.
‡Results compiled from references 2, 7, 14, and 16.

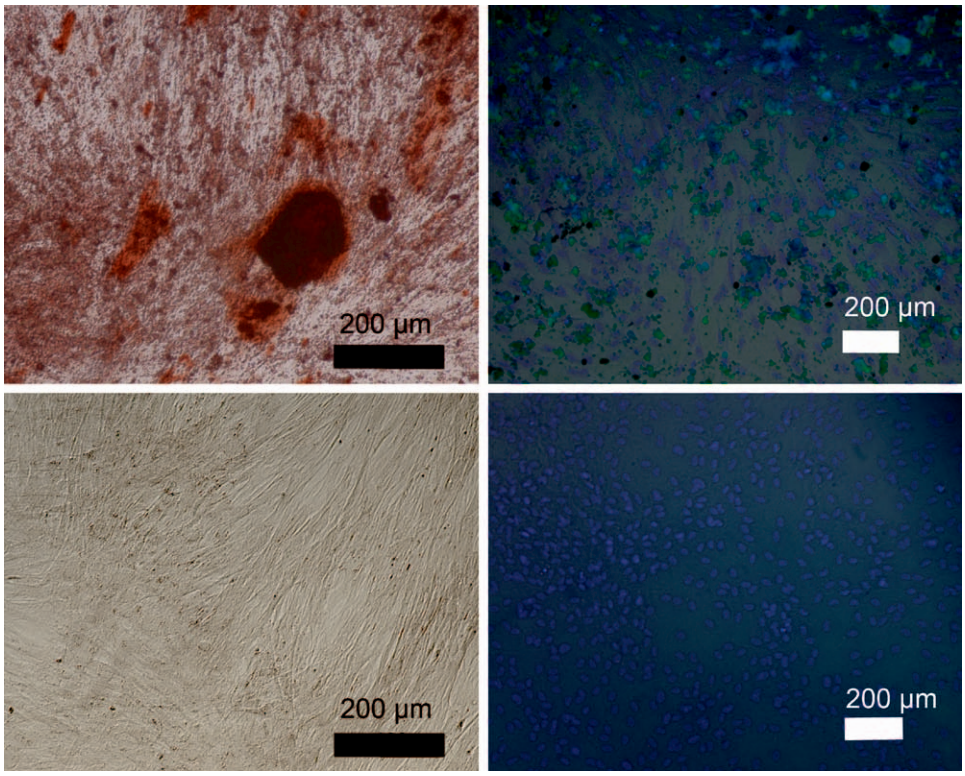

Fig. 3. Wharton jelly–derived mesenchymal stromal cells isolated and expanded with serum-free medium after 21 days of differentiation with osteogenic factors (*above*) and without them (control) (*below*). Positive staining for hydroxyapatite using alizarin (*above*, *left*) and OsteoImage (*above*, *right*) in the differentiation group and negative staining in the control group (*below, left* and *right*).

Alizarin red and OsteoImage (Fig. 3, *above*). Fresh and thawed samples showed equal mineralization performance.

The expression of osteogenic downstream genes increased after 3 weeks of osteodifferentiation (Fig. 4). The increase was significant for the early downstream genes *RUNX2* and *Hoxa10,* and a nonsignificant trend was observed for *OSX*, *ALPL*, *OCN*, and *BSP2*.

In Vivo Testing of Osteogenic Potential of Serum-free–Cultured Wharton Jelly–Derived Mesenchymal Stromal Cells

The intrinsic bone-forming capacity of naive human Wharton jelly–derived mesenchymal stromal cells was investigated by implanting constructs of hydroxyapatite granules and fibrin with and without Wharton jelly–derived mesenchymal stromal cells subcutaneously into immunoincompetent mice (Fig. 5). After 8 weeks, the cell-loaded constructs displayed a uniform tissue with a dense fibrillar extracellular matrix (Fig. 6, *above*, *left*), and all of the granules were surrounded by a dense continuous cell layer (Fig. 6, *above*, *center*). Control acellular constructs exhibited only loose connective tissue between the granules (Fig. 6, *below*, *left* and *center*). The staining for specific DNA human Alu sequences confirmed that the cells within the dense fibrillar matrix were derived from the human Wharton jelly–derived mesenchymal stromal cells (Fig. 6, *above*, *right*), whereas the staining was absent in acellular constructs (Fig. 6, *below*, *right*). The human cells within the matrix displayed spherical large nuclei (Fig. 6, *above*).

Direct Osteogenic Differentiation of Entire Wharton Jelly Biopsy Specimens

Because Wharton jelly itself is composed of collagen I, as with bone, the direct use of Wharton jelly biopsy specimens itself as a scaffold for osteodifferentiation is appealing and was therefore tested for such. The osteodifferentiated Wharton jelly biopsy specimens (Fig. 7, *above*) exhibited peripheral hydroxyapatite deposits confirmed by OsteoImage (Fig. 7, *above*, *left)* staining and von Kossa (Fig. 7, *above*, *center*), respectively, whereas it was absent in controls (Fig. 7, *below*, *left* and *center*). In every donor, 80 percent of the differentiated Wharton jelly biopsy specimens exhibited such calcifications.

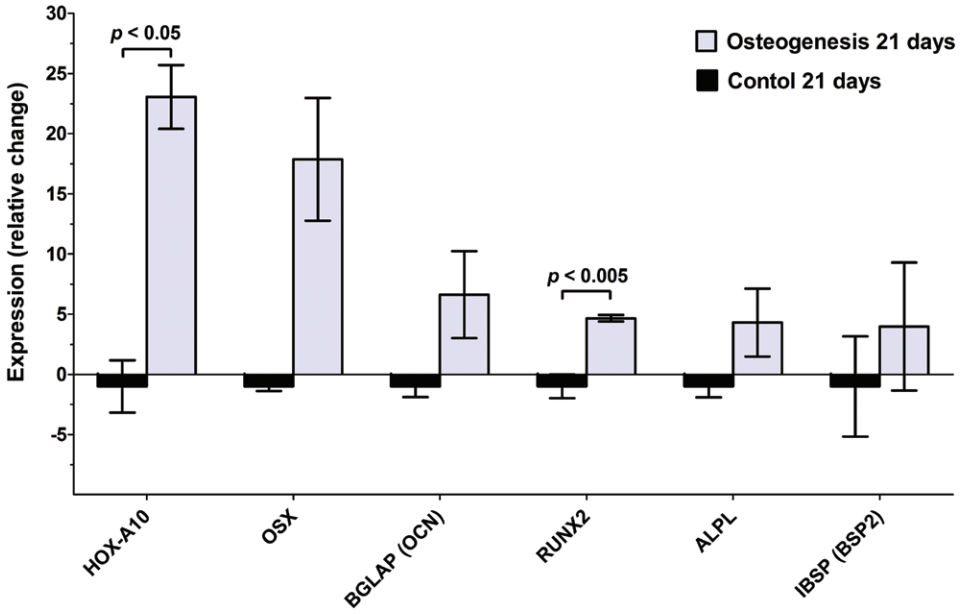

Fig. 4. Transcription profile of Wharton jelly–derived mesenchymal stromal cells isolated and expanded with a serum-free medium after 21 days of differentiation with osteogenic factors (*gray bars*), presented as mean ± SD values of the relative change in gene expression compared with the control condition without osteogenic factors (*black bars*). Transcription profiles of controls are normalized to −1 ($n = 4$; passages, ≤ 5). Gene, relative change, p value: *HOX-A10*, 23.1, $p < 0.04$; *OSX*, 17.9, $p < 0.16$; *BGLAP*, 6.6, $p < 0.19$; *RUNX2*, 4.7, $p < 0.005$; *ALPL*, 4.3, $p < 0.21$; and *IBSP*, 4.0, $p < 0.58$.

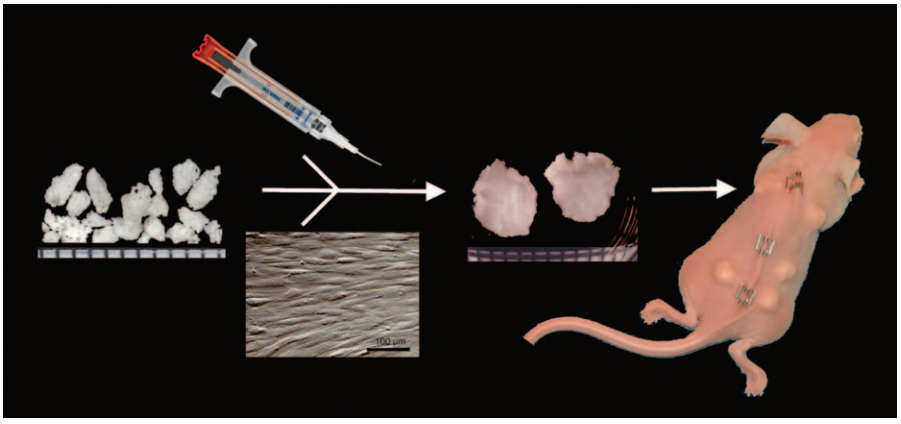

Fig. 5. Hydroxyapatite granules (*left*) were combined with Wharton jelly–derived mesenchymal stromal cells and fibrin (*second from left*) to produce osteogenic constructs (*second from right*). Constructs of hydroxyapatite granules and fibrin with and without human Wharton jelly–derived mesenchymal stromal cells were subcutaneously implanted in immunoincompetent mice for 8 weeks (*right*).

Staining for bone sialoprotein 2, which is a noncollagenous bone matrix protein produced by early committed osteogenic cells, was markedly increased in the periphery of osteogenic differentiated Wharton jelly biopsy specimens, with the intensity being maximal around the mineralization deposits (Fig. 7, *above*, *right*). In control specimens, bone sialoprotein 2 staining was negative (Fig. 7, *below*, *right*).

DISCUSSION

Wharton jelly–derived mesenchymal stromal cells are well protected in the umbilical cord and always survived for up to 48 hours at room temperature in the present study. This time window eases the clinical handling of Wharton jelly–derived mesenchymal stromal cells. The cord piece can be quickly obtained even in the rush of a delivery, and can later be processed for cryopreservation

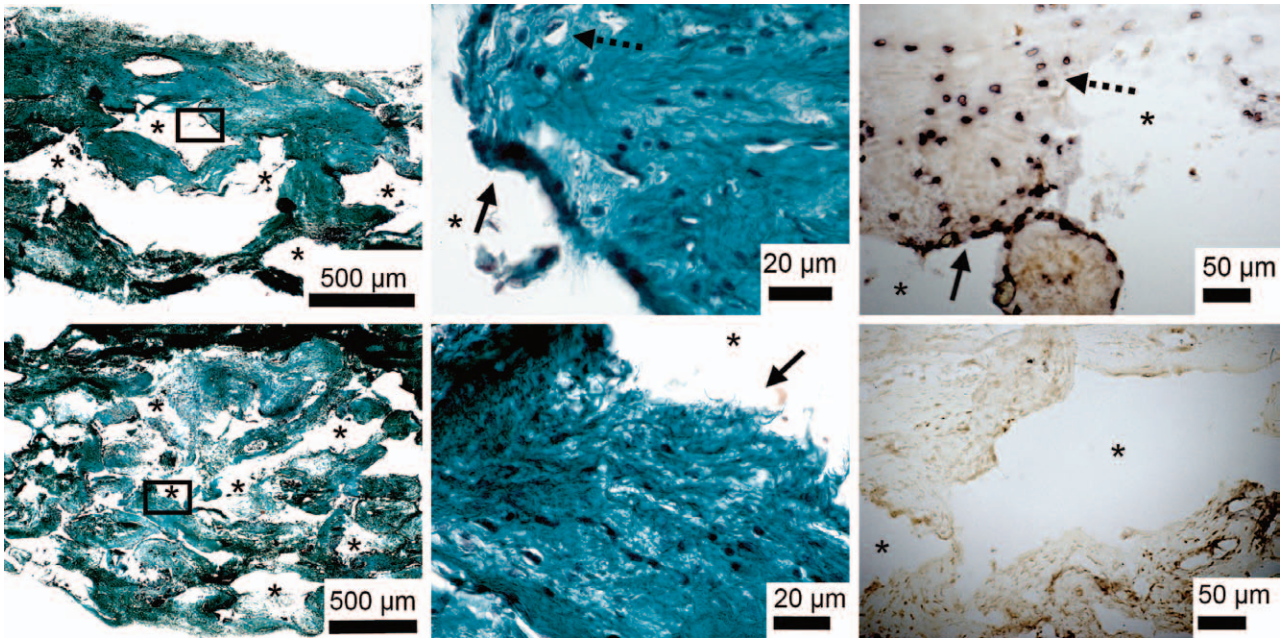

Fig. 6. Constructs of hydroxyapatite granules and fibrin with (*above*) and without (*below*) human Wharton jelly–derived mesenchymal stromal cells after 8 weeks of subcutaneous implantation in immunoincompetent mice. Wharton jelly–derived mesenchymal stromal cell–containing constructs formed a dense uniform tissue with fibrillar matrix (*above*, *left*) and the hydroxyapatite granules were surrounded by continuous cell layers (*solid arrow*; *above*, *center*, and *right*). In constructs without Wharton jelly–derived mesenchymal stromal cells, the tissue was irregular and loose (*below*, *left*), and no cell layer formed around the granules (*solid arrow*; *below*, *center*). Cells of human origin were identified by staining for human-specific Alu sequences. (*Above, right*) Human Wharton jelly–derived mesenchymal stromal cells are embedded within the dense lamellar matrices, as highlighted by the purple nucleus staining. The Wharton jelly–derived mesenchymal stromal cells showed spherical large nuclei (*dashed arrow*). Constructs devoid of human Wharton jelly–derived mesenchymal stromal cells were negative for human Alu sequence staining (*below*, *right*). *Lacunae of hydroxyapatite granules. (*Left* and *center*) Masson trichrome staining. (*Right*) Human-specific Alu sequences.

or transferred into a designated laboratory. Even storage at −80°C for up to at least 1 year appears to be possible; it is therefore feasible to temporarily store Wharton jelly–derived mesenchymal stromal cells in operating room freezers, which are commonly available for autologous bone grafting. The isolation technique allowed the removal of 32×10^6 Wharton jelly–derived mesenchymal stromal cells from a 10-cm cord piece at passage 1; this is sufficient for 10 constructs of 5-mm diameter, which allows the filling of cleft alveolar bone defects of any shape. To generate sufficient cell numbers for experimentation and intended clinical application, we cryopreserved and reexpanded the cells from each donor up to passages 3 to 5. No phenotypic changes were observed during the reexpansion. Wharton jelly–derived mesenchymal stromal cells have shown highly stable karyotype, population doubling time, and osteoblastic differentiation capacity up to passages 15 to 20.[15] Wharton jelly–derived mesenchymal stromal cells were even expanded beyond passage 40 without reaching senescence, whereas bone marrow mesenchymal stromal cells did so at passage 8.[17]

The Wharton jelly–derived mesenchymal stromal cell CD marker signature observed in the present study matched well the commonly described mesenchymal stromal cell phenotypes of adipose tissue and bone marrow, with the exception of immunostaining for CD106, which was negative in Wharton jelly–derived mesenchymal stromal cells, whereas in bone marrow mesenchymal stromal cells it is commonly expressed (Table 3). CD106 corresponds to vascular cell adhesion molecule 1, which mediates the adhesion of white blood cells and is not a mesenchymal-specific stem cell marker. Accordingly, in adipose tissue–derived mesenchymal stromal cells, CD106 is reportedly variably positive[2,6] or even negative.[15] Although defined CD marker signatures are commonly described for distinct mesenchymal stromal cell phenotypes, individual marker cannot predict the clinical performance of bone formation. Thus, regardless of the question as to whether Wharton jelly–derived mesenchymal stromal cells are true mesenchymal stromal cells,[7] their ability to form bone remains to be established.

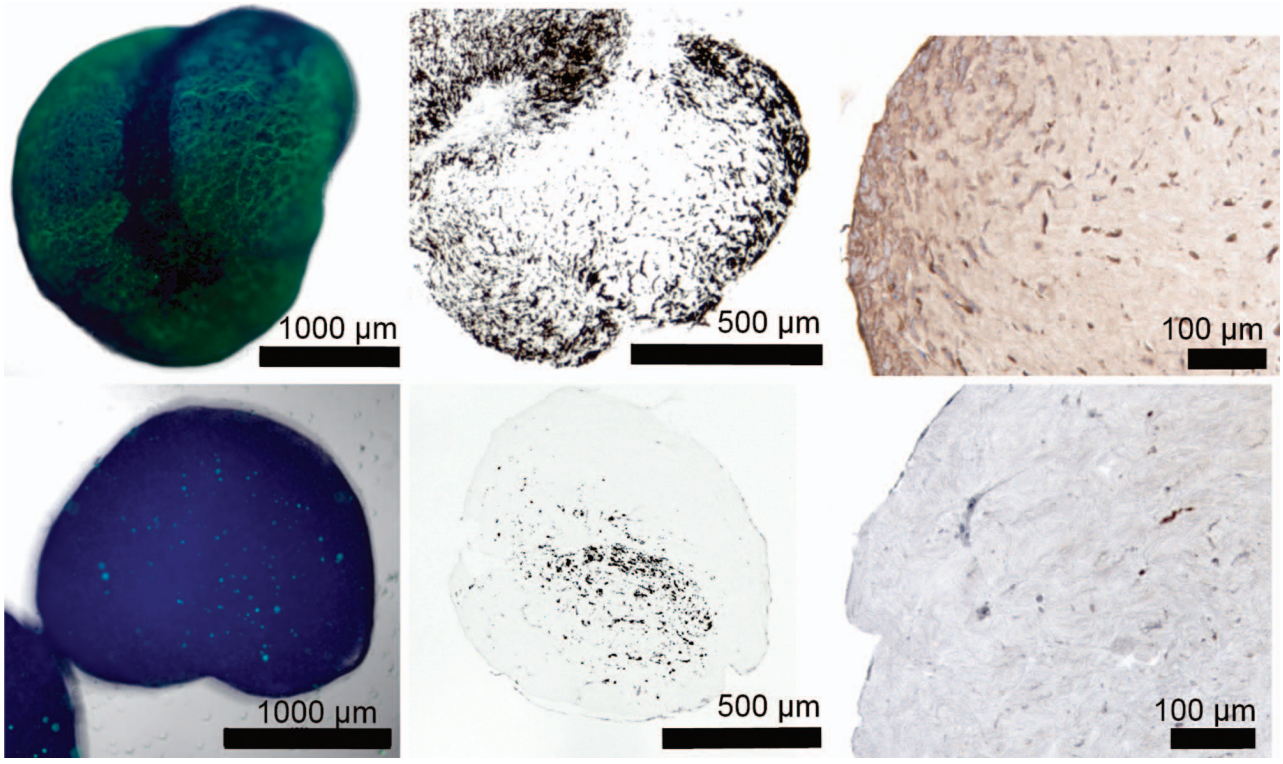

Fig. 7. Wharton jelly biopsy specimens after 21 days of in vitro differentiation with (*above*) or without osteogenic factors (*below*). Mineralization assay on native Wharton jelly biopsy specimens showing peripheral net-like hydroxyapatite staining on osteodifferentiated samples (*above*, *left*) and absence in the control samples (*below*, *left*). Von Kossa stain confirming strong peripheral mineralization in differentiated Wharton jelly biopsy specimens (*above*, *center*) and weak central mineralization in undifferentiated samples (*below*, *center*). The bone sialoprotein 2 staining intensity (*above*, *right*) was maximal around the hydroxyapatite deposits (*above*, *right*). Control samples without osteogenic factors were negative for bone sialoprotein 2 (*below*, *right*).

It was assumed that mesenchymal stromal cells from neonatal tissue are more naive[6] and have a greater proliferation capacity and differentiation potential[18] relative to bone marrow mesenchymal stromal cells, which are derived from adult tissue. Wharton jelly–derived mesenchymal stromal cells also reportedly exhibit a greater bone-formation capacity compared with adipose-derived stem cells,[19] making them a promising cell source for the correction of bone defects related to congenital malformations. However, one limitation is the absence of a method for growing the cells under a fully defined and regulated protocol.

Osteodifferentiated Wharton jelly–derived mesenchymal stromal cells exhibited characteristic mineralization in monolayers. Thus, the osteodifferentiation ability of Wharton jelly–derived mesenchymal stromal cells withstands the serum-free cultivation method. In line with the histology, the expression of early and specific genes downstream from osteogenesis increased significantly under osteogenesis: the expression of *RUNX2* increased and up-regulated the transcription of *OSX* and *ALPL*, and the expression of *Hoxa10* increased, which is a functional element in osteoblast genes, including *OCN*, *BSP2*, and *ALPL*.[20]

The spontaneous in vivo bone-forming capacity of Wharton jelly–derived mesenchymal stromal cells was tested by subcutaneous implantation of constructs that contained naive Wharton jelly–derived mesenchymal stromal cells. Thus, despite the absence of bone signaling and the absence of predifferentiation, the Wharton jelly–derived mesenchymal stromal cells started to produce a dense unmineralized lamellar matrix, similar to bone matrix, around the hydroxyapatite granules. The therein embedded Wharton jelly–derived mesenchymal stromal cells displayed large spherical nuclei, resembling osteoblastic lineage cells (Fig. 6). However, the differentiation was not sufficient to produce woven or lamellar bone within an 8-week period.

The data in the literature regarding the ability of Wharton jelly–derived mesenchymal stromal cells to produce ectopic bone are inconsistent. Although mature bone formation has been reported for predifferentiated Wharton jelly–derived mesenchymal stromal cells on

hydroxyapatite–polylactic acid scaffolds after 12 weeks,[8] others have found a solely mineralized matrix, but no mature bone, when using predifferentiated polycaprolactone/tricalcium phosphate/Wharton jelly–derived mesenchymal stromal cells constructs.[19] Nonetheless, predifferentiation of Wharton jelly–derived mesenchymal stromal cells might critically enhance their ability to form bone in vivo. Predifferentiation has also been applied to tests of orthotopic bone formation by Wharton jelly–derived mesenchymal stromal cells.[9,10] In vitro predifferentiation is commonly performed with bovine serum, which is difficult to replace with serum-free components. In the future, autoserum from the cord blood[21] or extract from umbilical cord tissue[22] could be an option to satisfy autologous and clinical translatable conditions.

Osteodifferentiated Wharton jelly biopsy specimens exhibited hydroxyapatite mineralization in the Wharton jelly tissue, which we attribute to osteodifferentiation of Wharton jelly–derived mesenchymal stromal cells within their natural niche. Bone sialoprotein is a major noncollagenous glycoprotein of the bone matrix that promotes the initiation of mineralization. Accordingly, it was found in maximal concentrations around the hydroxyapatite deposits. To our knowledge, this is the first report on the osteogenic differentiation of native Wharton jelly tissue. The structural similarity between the extracellular matrix of Wharton jelly and bone, both comprising collagen I with coassembled collagen V,[23] might be an advantage for bone or periosteal tissue engineering. For cleft surgical application, frozen and thawed Wharton jelly tissue specimens could be evaluated as an autologous viable and periost-like material for palatal fistula repair, for which amnion,[24] acellular dermal matrix,[25] and autologous cancellous bone[26] have been proposed. Even more promising, the Wharton jelly tissue could be an alternative to free tissue grafts for primary cleft palate surgery.[27] Free tissue grafts have been proposed in palatal surgery as a reinforcement onto vomerine flaps, to avoid lateral bone denudation[28,29]—or as a soft-tissue graft onto laterally denuded bone,[30] to avoid healing by secondary intention, which inhibits palatal growth.[31]

CONCLUSIONS

We present in this article an in vivo protocol in which Wharton jelly–derived mesenchymal stromal cells exhibit signs of osteogenic commitment and form a dense collagenous matrix under serum-free conditions and that is compatible with clinical translation. A striking finding of our work concerned the conversion of Wharton jelly tissue into mineralized tissue under osteogenetic differentiation. This raises new options for bone-regenerative techniques from the umbilical cord, including direct transplantation of umbilical cord tissue graft for primary cleft palate surgery.

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REFERENCES

1. Goldring CE, Duffy PA, Benvenisty N, et al. Assessing the safety of stem cell therapeutics. *Cell Stem Cell* 2011;8:618–628.
2. García-Gómez I, Elvira G, Zapata AG, et al. Mesenchymal stem cells: Biological properties and clinical applications. *Expert Opin Biol Ther.* 2010;10:1453–1468.
3. Ishige I, Nagamura-Inoue T, Honda MJ, et al. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. *Int J Hematol.* 2009;90:261–269.
4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408.
5. Güven S, Mehrkens A, Saxer F, et al. Engineering of large osteogenic grafts with rapid engraftment capacity using mesenchymal and endothelial progenitors from human adipose tissue. *Biomaterials* 2011;32:5801–5809.
6. Nekanti U, Rao VB, Bahirvani AG, Jan M, Totey S, Ta M. Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. *Stem Cells Dev.* 2010;19:117–130.
7. Bosch J, Houben AP, Radke TF, et al. Distinct differentiation potential of "MSC" derived from cord blood and umbilical cord: Are cord-derived cells true mesenchymal stromal cells? *Stem Cells Dev.* 2012;21:1977–1988.
8. Diao Y, Ma Q, Cui F, Zhong Y. Human umbilical cord mesenchymal stem cells: Osteogenesis in vivo as seed cells for bone tissue engineering. *J Biomed Mater Res A* 2009;91:123–131.
9. Kuo HC, Chiu CC, Chang WC, et al. Use of proteomic differential displays to assess functional discrepancies and adjustments of human bone marrow- and Wharton jelly-derived mesenchymal stem cells. *J Proteome Res.* 2011;10:1305–1315.
10. Wen Y, Jiang B, Cui J, et al. Superior osteogenic capacity of different mesenchymal stem cells for bone tissue

engineering. *Oral Surg Oral Med Oral Pathol Oral Radiol.* 2013;116:e324–e332.
11. La Rocca G, Anzalone R, Corrao S, et al. Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: Differentiation potential and detection of new markers. *Histochem Cell Biol.* 2009;131:267–282.
12. Nekanti U, Dastidar S, Venugopal P, Totey S, Ta M. Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia. *Int J Biol Sci.* 2010;6:499–512.
13. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. *Stem Cells* 2005;23:220–229.
14. Al-Nbaheen M, Vishnubalaji R, Ali D, et al. Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential. *Stem Cell Rev.* 2013;9:32–43.
15. Folgiero V, Migliano E, Tedesco M, et al. Purification and characterization of adipose-derived stem cells from patients with lipoaspirate transplant. *Cell Transplant.* 2010;19:1225–1235.
16. Mafi P, Hindocha S, Mafi R, Griffin M, Khan WS. Adult mesenchymal stem cells and cell surface characterization: A systematic review of the literature. *Open Orthop J.* 2011;5(Suppl 2):253–260.
17. Lund RD, Wang S, Lu B, et al. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells* 2007;25:602–611.
18. Hsieh JY, Fu YS, Chang SJ, Tsuang YH, Wang HW. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. *Stem Cells Dev.* 2010;19:1895–1910.
19. Zhang ZY, Teoh SH, Chong MS, et al. Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells* 2009;27:126–137.
20. Hassan MQ, Tare R, Lee SH, et al. HOXA10 controls osteoblastogenesis by directly activating bone regulatory and phenotypic genes. *Mol Cell Biol.* 2007;27:3337–3352.
21. Baba K, Yamazaki Y, Ikemoto S, Aoyagi K, Takeda A, Uchinuma E. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived autoserum. *J Craniomaxillofac Surg.* 2012;40:768–772.
22. Kim SM, Moon SH, Lee Y, Kim GJ, Chung HM, Choi YS. Alternative serum-free biomaterials derived from human umbilical cord for the self-renewal ex-vivo expansion of mesenchymal stem cells. *Stem Cells Dev.* 2013;22:3025–3038.
23. Franc S, Rousseau JC, Garrone R, van der Rest M, Moradi-Améli M. Microfibrillar composition of umbilical cord matrix: Characterization of fibrillin, collagen VI and intact collagen V. *Placenta* 1998;19:95–104.
24. Rohleder NH, Loeffelbein DJ, Feistl W, et al. Repair of oronasal fistulae by interposition of multilayered amniotic membrane allograft. *Plast Reconstr Surg.* 2013;132:172–181.
25. Losee JE, Smith DM, Afifi AM, et al. A successful algorithm for limiting postoperative fistulae following palatal procedures in the patient with orofacial clefting. *Plast Reconstr Surg.* 2008;122:544–554.
26. Martin-Smith JD, O'Sullivan JB, Duggan L, O'Mahony A, Orr DJ. Repair of anterior cleft palate fistulae with cancellous bone graft: A simple technique that facilitates dental reconstruction. *Plast Reconstr Surg.* 2013;131:380e–387e.
27. Huang L, Wong YP, Gu H, et al. Stem cell-like properties of human umbilical cord lining epithelial cells and the potential for epidermal reconstitution. *Cytotherapy* 2011;13:145–155.
28. Akamatsu T, Tanino R, Osada M, Sakuma Y. Influences of different palatoplasties on palatal growth and speech development: Comparison between Osada's two-stage palatoplasty and one-stage mucosal flap procedure. *Tokai J Exp Clin Med.* 2004;29:111–122.
29. Neiva C, Dakpe S, Gbaguidi C, Testelin S, Devauchelle B. Calvarial periosteal graft for second-stage cleft palate surgery: A preliminary report. *J Craniomaxillofac Surg.* (in press).
30. Meng T, Shi B, Lu DW, Li Y, Wu M. Roles of palatine bone denudation repairing with free buccal or palatal mucosal graft on maxillary growth: An experimental study in rabbits. *Ann Plast Surg.* 2007;59:323–328.
31. Bardach J, Kelly KM. Does interference with mucoperiosteum and palatal bone affect craniofacial growth? An experimental study in beagles. *Plast Reconstr Surg.* 1990;86:1093–1100; discussion 1101.