Differentiation of Human Skeletal Muscle Stem Cells into Odontoblasts Is Dependent on Induction of α1 Integrin Expression*

Received for publication, October 11, 2013, and in revised form, March 14, 2014. Published, JBC Papers in Press, April 1, 2014, DOI 10.1074/jbc.M113.526772

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Background: Human skeletal muscle stem cells (hSMSCs) can differentiate into bone and fat cells.

Results: hSMSCs could also differentiate into odontoblasts but required an extracellular matrix scaffold and changes in their integrin profile.

Conclusion: The present results identify an odontoblast differentiation pathway dependent on adhesion receptor expression.

Significance: The data provide insight into how hSMSC adhesion receptors interact with the microenvironment to regulate lineage specification.

Skeletal muscle stem cells represent an abundant source of autologous cells with potential for regenerative medicine that can be directed to differentiate into multiple lineages including osteoblasts and adipocytes. In the current study, we found that α7 integrin-positive human skeletal muscle stem cells (α7+hSMSCs) could differentiate into the odontoblast lineage under specific inductive conditions in response to bone morphogenetic protein-4 (BMP-4). Cell aggregates of FACS-harvested α7+hSMSCs were treated in suspension with retinoic acid followed by culture on a gelatin scaffold in the presence of BMP-4. Following this protocol, α7+hSMSCs were induced to down-regulate myogenic genes (MYOD and α7 integrin) and up-regulate odontogenic markers including dentin sialophosphoprotein, matrix metalloproteinase-20 (enamelysin), dentin sialoprotein, and alkaline phosphatase but not osteoblastic genes. Following retinoic acid and gelatin scaffold/BMP-4 treatment, there was a coordinated down-regulation of muscle-specific α7β1 integrin expression. Interestingly, using siRNA knockdown strategies revealed that the differentiation-related expression of the α1 integrin receptor positively regulates the expression of the odontoblastic markers dentin sialophosphoprotein and matrix metalloproteinase-20. These results strongly suggest that the differentiation of α7+hSMSCs along the odontogenic lineage is dependent on the concurrent expression of α1 integrin.

One of the main goals of regenerative medicine is to repair wounds or regenerate the injured organs with autogenic stem cells so that there are minimal immunological rejections. Stem cell-based tooth regeneration is a promising approach to solving the problems of tooth loss. Previous studies have demonstrated that tooth germ cells and dental pulp stem cells from adult or deciduous tooth have the potential to differentiate into odontoblast lineages and undergo dentinogenesis (1–4). However, these cells cannot meet all of the clinical requirements. For example, cells of tooth origin cannot be obtained from edentulous jaw. Therefore, it is necessary to explore the potential of nondental autogenic cells that can be used as candidates for tooth regeneration. Evidence accumulated from many studies (5–7) suggests that nondental ectomesenchymal cells such as that from the second pharyngeal arch or the first bronchial arch contain an odontogenic potentiality. These cells can also be replaced by cultured nondental mesenchymal stem cells from bone marrow (8). In addition, mesenchymal stem cells are found in other adult tissues such as adipose tissues and hair follicles, which are more superficial and easily accessible than bone marrow. Interestingly, morphogenesis of hair follicle as well as tooth initiates from epithelial-mesenchymal interactions (9, 10). Unique to the hair follicle, this reciprocal epithelial-mesenchymal cross-talk persists from embryo stages to adulthood, resulting in the dynamic and continuous growth of a normal follicle (11). Several studies have examined the ability of dental pulp stem cells to regenerate dentin (12, 13). There exists the possibility of effective therapies involving stem cell injection to take the place of usual dental cavity treatment and pulp capping methods.

We previously isolated human fetal and satellite muscle stem cells by taking advantage of the high expression of the α7 integrin as a marker for the myogenic lineage. The α7-positive fetal cells were capable of fusion and could differentiate into myotubes with high efficiency; therefore, fetal human cells can be easily purified and expanded in vitro to obtain large numbers of...
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The present study was designed to investigate the odontogenic potential of α7+ multipotent muscle stem cells from human skeletal muscle stem cells. We have examined the potential of human fetal myogenic cells to differentiate along the odontogenic pathway and defined how adhesion and migration are modulated during this process. Our results demonstrated for the first time that human skeletal muscle stem cells can differentiate into odontoblast-like cells and may be useful as a strategy for tooth regeneration. In addition, evidence is provided that indicates the up-regulation of a specific α1 integrin is a necessary step in the conversion of myogenic stem cells to odontoblast lineage.

EXPERIMENTAL PROCEDURES

Cells and Culture—The α7 integrin-positive human skeletal muscle stem cells (α7+-hSMSCs) were isolated from fetal tongue (14–24 weeks prenatal) and maintained as described previously (14) with minor modifications. In brief, cells (passage 6–8) were cultured in Ham’s F-10 medium (Invitrogen) containing 20% fetal bovine serum (Invitrogen), 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 μg/ml insulin (Invitrogen), 2.5 μg/ml Fungizone (Invitrogen), 0.5 μg/ml gentamicin (Invitrogen), and 2 mM L-glutamine (Invitrogen). Rat odontoblast-like cells (KN-3; kindly provided by Dr. Chiaki Kitamura, Kyushu Dental College, Kitakyushu, Japan) were maintained as described previously (15). Mouse odontoblast-like cell line MC3T3-E1 was obtained from the Riken cell bank and cultured in plastic dishes containing minimal essential medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C in air with 5% CO2 and then subcultured until almost confluent (16, 17). This study was approved by the University of California, San Francisco Committee on Human Research and Aichi Gakuin University Ethics Committee (Approval Number 82).

Odontogenic Differentiation—The formation of embryoid body-like structures with α7+-hSMSCs was carried out using a hanging drop method based on a protocol described previously (18). Cell aggregates were pooled on non-adherent bacterial containing 20% fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a 5% CO2 humidified incubator, and the medium was changed every other day. At the end of 7 days of incubation, cells in the lower chamber were harvested by detachment with 3 mM EDTA in phosphate-buffered saline (PBS). The experimental protocol used is depicted in Fig. 1. Purified osteoblast cells derived from α7+-hSMSCs were prepared as reported previously (14).

In brief, the α7+-hSMSC-derived osteoblasts were prepared and maintained as described previously (14) with minor modifications. In brief, cells were cultured in Ham’s F-10 medium (Invitrogen) containing 20% fetal bovine serum (Invitrogen), 300 ng/ml BMP-2 (Peprotech Inc.), 50 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 1 mg/ml insulin (Invitrogen), 2.5 μg/ml Fungizone (Invitrogen), 0.5 μg/ml gentamicin (Invitrogen), and 2 mM L-glutamine (Invitrogen). Previous studies show that the monoclonal anti-α2 integrin antibody can potently suppress the expression of osteoblastic markers in the described culture system (14), and we confirmed that the expression of α2 integrin in α7+-hSMSCs triggered their differentiation into osteoblast-like cells. Therefore, using fluorescence-activated cell sorting (FACS), we evaluated the ratio of α2 integrin-positive cells as a percentage of the total differentiated cells as a means to determine the purity of the differentiated cell population. We found that our cultures of α7+-hSMSC-derived osteoblast cells were 98.41 ± 1.59% homogenous (percent total, n = 3). We also detected the expression of the osteoblastic markers osteopontin and osteocalcin in α7+-hSMSC-derived osteoblast cells, presenting further evidence that the α7+-hSMSCs had differentiated along the osteoblastic lineage (data not shown). We also confirmed specific osteogenic physiological changes in the cells (e.g. calcification and increased alkaline phosphatase activity) that continued over a period of 21 days (data not shown).

Cell Proliferation Assay—To develop a suitable scaffold that promoted growth and differentiation, we tested several extracellular matrix proteins including collagen type I (PureCol Collagen, Advanced BioMatrix, Inc., San Diego, CA), collagen type IV (PureCol Collagen, Advanced BioMatrix, Inc.), fibronectin (BD Biosciences), and gelatin (Sigma-Aldrich). The cells were preincubated as a hanging drop culture to form aggregates, then treated with RA, and grown in 96-well tissue culture plates at a density of 1 × 10^5 cells/cm². Extracellular matrix (ECM) proteins at a suitable concentration were added as soluble proteins to the growth medium for the indicated time, and then cell proliferation was evaluated using a BrdU cell proliferation enzyme-linked immunosorbent assay (ELISA) (Roche Applied Science) as described previously (16, 17).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—The protocol for RT-PCR has been described previously (19). The PCR within the exponential phase of the amplification curve was performed for 25 cycles for MYOD (muscle-specific marker); FOXD3 and SOX10 (neural crest markers); MEOXI (presomitic mesoderm marker); RUNX2 (osteogenic transcription factor); DSPP, dentin matrix protein 1 (DMP-1), and MDP-20 (odontoblast markers); osteopontin and osteocalcin (osteoblast markers); and glyceraldehyde-3-phosphate dehy-

2 The abbreviations used are: α7+-hSMSC, α7 integrin-positive human skeletal muscle stem cell; BMP, bone morphogenetic protein; RA, retinoic acid; GS, gelatin scaffold; DSP, dentin sialophosphoprotein; MMP, matrix metalloproteinase; DSP, dentin sialoprotein; EB, embryoid body; ECM, extracellular matrix; ALPase, alkaline phosphatase; ARS, Alizarin Red S; Coll, collagen; DMP-1, dentin matrix protein 1.
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drogenase (GAPDH) in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The following primer sequences were used (NCBI Reference Sequence accession numbers are given): human MYOD (sense, 5'-gtcggacgcat-gatgga-3'; antisense, 5'-cggcctgttcctgtcctg-3'; 106-bp amplion; NM_002478), human FOXD3 (sense, 5'-ctcagctggatacctcagcaac-3'; antisense, 5'-cgaacatgccgctggactgcg-3'; 180-bp amplion; NM_012183), human SOX10 (sense, 5'-cccccagctgattggaaccc-3'; antisense, 5'-cctgctggctttgctttcct-3'; 80-bp amplion; NM_006941), human ME0X1 (sense, 5'-aactgggaccttctgcatac-3'; antisense, 5'-cgcctgtggatgtttcttctctg-3'; 173-bp amplion; NM_004527), human DSPP (sense, 5'-tcctttggaagccttttaagccatg-3'; antisense, 5'-tgggtttgtctggagtaagctggagtaat-3'; 117-bp amplion; NM_014208), human MMP-20 (sense, 5'-ctcttcaggaagccctatcctc-3'; antisense, 5'-cactttctctctctctctcta-3'; 194-bp amplion; NM_004771), human integrin α1 (sense, 5'-aanccgtccagccaattctgactg-3'; antisense, 5'-ttactccggagttctctctctctctctc-3'; 173-bp amplion; NM_181501), and human GAPDH (sense, 5'-cagcaacagtctggactgcg-3'; antisense, 5'-ggcaacaatctacccctct-3'; 153-bp amplion; M_002046). Band density was scanned and evaluated by MultiGauge Version 3.X (Fujifilm, Tokyo, Japan).

Real Time Quantitative PCR Analysis—Real time quantitative PCR reactions for all samples and standards were performed in triplicate in 96-well microtiter plates with approximately 25 ng of RNA, 0.25 µl of the RT mixture (Qiagen QuantiTect RT Mix, Qiagen Inc., Valencia, CA), 1.25 µl of the 20× Primer/Probe Mix (human MYOD (MYOD1), Hs00230075_g1; human DSPP, Hs00171962_m1; human DMP-1, Hs01009390_m1; human MMP-20, Hs01573770_m1; human osteopontin (SPP1), Hs00959010_m1; human osteocalcin (BGLAP), Hs00609452_g1; Assays On Demand, Applied Biosystems) and 12.5 µl of the Mastermix (Qiagen QuantiTect RT-PCR kit) in a 25-µl reaction volume. Standards and samples were mixed with the PCR reagents, loaded onto the 96-well microtiter plate, and sealed with optical film (Applied Biosystems). TaqMan samples were subjected to thermal cycling conditions with the following parameters: an initial holding stage of 30 min at 50 °C for RNA to be reverse transcribed and 15 min at 95 °C to activate the HotStar Taq polymerase enzyme followed by 40 cycles of 15 s at 94 °C and then 60 s at 60 °C. The standard curve method was used to determine the relative quantification of gene expression, whereas GAPDH and 18 S ampolion rRNA were used as housekeeping genes and as a control to normalize for variations in the amount of total RNA in each sample. For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curve, and then the target amount was divided by the endogenous reference amount to obtain a normalized target value. Ct (threshold cycle values) for samples and housekeeping genes were extrapolated across the standard curve to produce an arbitrary value of expression, the ratio of which (sample/housekeeping gene) within a given sample was plotted as the relative mRNA expression.

Western Blotting—Total protein extracts were prepared from cells that were cultured for 12 days in the presence or absence of RA + GS/BMP-4. Lysates were separated on 12% SDS-polyacrylamide gels and analyzed by Western blotting using anti-MYOD (sc-760), anti-FOXD3 (sc-13358), anti-SOX10 (sc-17342), anti-MEOX1 (sc-134389), anti-DSP (sc-73632), anti-MMP-20 (sc-18328), anti-dentin sialoglycoprotein (DSP) (sc-18328), GAPDH (sc-25778), and β-tubulin (sc-9104) polyclonal antibodies (Santa Cruz Biotechnol, Inc., Santa Cruz, CA). No significant cross-reactivity was observed with other proteins tested (data not shown).

Cells were transfected with control, nontargeting siRNA, or siRNA specific for α1 integrin for the specified times. Cells were then processed for SDS-PAGE, and protein was transferred to nitrocellulose paper, blocked, processed with primary antibodies (anti-α1 integrin (sc-10728), anti-α1 integrin (sc-53711), and anti-β-tubulin (sc-9935), respectively (Santa Cruz Biotechnology, Inc.) followed by incubation with secondary antibodies conjugated to peroxidase, and finally developed by ECL Plus Western Blotting Detection Reagent (Amersham Biosciences). Visualization and quantification of blotted protein band densities were performed with MultiGauge Version 3.X (Fujifilm). ELISA—Commercially available ELISAs for human MYOD (AMS.E01M00233, AMS Biotechnology Ltd., Abingdon, UK), human DSPP (CSB-EL007209HU, CUSABIO Biotech, Co., Ltd. Wuhan, China), human DMP-1 (CSB-E13029h, CUSABIO Biotech, Co., Ltd.), human osteopontin (ab100618, Abcam, Cambridge, UK), and human osteocalcin (KQA1381, Invitrogen) were performed to determine the content of each protein according to the each manufacturers’ instructions.

Immunofluorescence Microscopy—Immunofluorescence staining was carried out as described previously (14). Cells were incubated with primary antibody against DSP (2 µg/ml; sc-18325, Santa Cruz Biotechnology, Inc.) followed by staining with fluorescein isothiocyanate (FITC)-labeled anti-goat IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and cell nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen). Stained samples were imaged using a BZ-9000 microscope (Keyence, Osaka, Japan).

Alkaline Phosphatase (ALPase) Activity Assay—Cellular ALPase activity was measured using p-nitrophenol phosphate (Sigma-Aldrich) as a substrate. Briefly, following washing, the cells were incubated in 150 µl of ALPase buffer (Sigma-Aldrich) and 150 µl of p-nitrophenol phosphate solution at 37 °C for 30 min with gentle shaking in the dark. Subsequently, 700 µl of 3 M NaOH solution was added to each well to stop the reaction, and then 100 µl of the final solution was placed into a 96-well plate well and read at 405 nm in an ELISA microplate reader (SH-1200 Lab, Corona Electric Co., Ltd., Ibaraki, Japan). The enzyme activity was calibrated with a p-nitrophenol phosphate standard curve and expressed as micromoles of reaction product/minute/total protein obtained from the protein quantification in each well.

Alizarin Red S (ARS) Staining and Quantification—Mineralization from the embryonic stem cell-derived osteogenic cells was quantified using the ARS assay. Briefly, following washing, the cells were immersed in a 40 mM ARS (Sigma-Aldrich) solution (pH 4.2) for 20 min at room temperature with gentle agitation. The solution then was removed, and the mineralized matrices were washed with flowing water. The morphology of
mineralized matrices was observed and photographed using a BZ-9000 microscope (Keyence). ARS staining was quantified using the method developed by Gregory et al. (20). Briefly, following staining and washing, the samples were dried overnight, and then 0.8 ml of 10% (v/v) acetic acid (VWR International, Radnor, PA) was added to each well followed by incubation at room temperature for 30 min with shaking. The loosely attached cells were scraped with a cell scraper, transferred to microcentrifuge tubes, and vortexed for 30 s. Samples were heated at 85 °C for 10 min followed by cooling in ice for 5 min. After centrifugation at 20,000 × g for 15 min, 200 μl of 10% (v/v) ammonium hydroxide (Sigma-Aldrich) was added. Finally, 100 μl of the supernatant was placed in a 96-well plate and measured at 405 nm with an ELISA microplate reader (SH-1200 Lab).

RNA Interference and Function-blocking Antibody Targeting of α1 Integrin—To assess whether expression of α1 integrin is an essential and permissive event for odontoblastic differentiation, two approaches were used to disrupt integrin expression or function. The anti-α1 integrin siRNA for gene silencing was acquired commercially (Santa Cruz Biotechnology, Inc.) and transfected into cultured cells using the siRNA Reagent System (Santa Cruz Biotechnology, Inc.) according to the manufacturer’s protocol. GAPDH siRNA and a scrambled siRNA with no known homology for any vertebrate sequence (Thermo Scientific, Lafayette, CO) were used as positive and negative controls, respectively. EB formation from α7+ hSMSCs was carried out as described above followed by culture as cell aggregates with RA for 2 days. Then the RA-treated cells were plated as adherent cultures and processed for siRNA transfection or treatment with anti-integrin monoclonal antibody (mAb). For siRNA transfection, 2 × 10⁵ cells were transfected with 66 pmol of siRNA using the siRNA Reagent System (Santa Cruz Biotechnology, Inc.) for 24 h. For function-blocking antibody treatment, TS2/7 mAb (Abcam) (22, 23) was used at 5 µg/ml for 1 h. After 1-h incubation, the cells were treated with GS/BMP-4 for 7 days and then were tested for differentiation status.

Flow Cytometry—Flow cytometry was performed using standard procedures (14) with the following mAbs: anti-human integrin α1 (TS2/7, mouse monoclonal, Abcam) (21, 22), mouse anti-human integrin α2 (P1E6, Abcam), mouse anti-human integrin α5 (P1D6, Abcam), rat anti-human integrin α6 (GoH3, Abcam), mouse anti-human integrin α7 (9.1; custom made as described previously (23)), mouse anti-human integrin αV (P1F6, Abcam), and mouse anti-human integrin αV/β3 (VNR-1, Abcam).

Cell Adhesion and Migration Assay—Analysis of cell adhesion was performed on ligand-coated wells as described previously (14, 19). Single cell suspensions were incubated for 20 min in 96-well plates coated with laminin-1 (5 µg/ml; laminin 111, Chemicon, Temecula, CA) or 30 min on collagen type I (Col-I) (1 µg/ml; PureCol Collagen, Advanced BioMatrix, Inc.) at 37 °C. Cell migration was assayed as described previously (24). The undersides of the Transwell (8-µm pore) were precoated with laminin 111 (5 µg/ml) or Col-I (1 µg/ml). The cells that migrated through the filter were counted and averaged from 10 randomly chosen microscopic fields using a 20× objective.

Statistics—All data are reported as the mean ± S.D. Statistical significance was assessed using the Mann-Whitney U test. p values <0.05 were considered statistically significant.

RESULTS

Optimization of Odontogenic Differentiation of α7⁺ hSMSCs—Effect of Scaffold—Previous analysis showed that α7⁺ hSMSCs expanded from both fetal and adult human muscle consistently expressed muscle lineage markers including MyoD and are highly capable of going through multiple rounds of proliferation before terminally differentiating to form myotubes (25). To explore the potential of α7⁺ hSMSCs to lose their myogenic lineage-committed phenotype and differentiate into odontoblast-like cells, we initially examined the influence of diverse ECM substrates to support the growth and differentiation of α7⁺ stem cells into odontoblast lineage following the differentiation protocol (Fig. 1). The cells were first preincubated in hanging drop culture to form EB-like aggregates and then treated with RA to induce differentiation. We analyzed several ECM molecules including Col-I, fibronectin, Col-IV, and gelatin. As evaluated by cell proliferation assays, we found that the GS was the most suitable as an ECM substrate (Fig. 2A). Next, we tested the capacity of the GS to induce proliferation of cells cultured as EBs derived from α7⁺ hSMSCs. Cells were cultured on gelatin substrates for 14 days and then examined for cell proliferation using a BrdU-based cell growth ELISA. It appears that 15% gelatin was optimal for cell growth (Fig. 2B; *, p < 0.05; ***, p < 0.01).

To assess their potential for odontogenic differentiation, we evaluated the response of α7⁺ hSMSCs to several TGF-β superfamily growth factors including BMP-2, -4, and -7. Following the differentiation protocol, samples were assessed by real time PCR for differentiation markers. When EB-like aggregates were cultured with GS combined with BMP-2 (300 ng/ml), BMP-4 (100 ng/ml), or BMP-7 (100 ng/ml) for 7 days, only BMP-4-treated cells showed higher expression of DSPP and DMP-1 mRNA as odontoblast markers compared with BMP-2- and BMP-7-treated cells (Fig. 2C). However, BMP-2- but not BMP-4-treated cells showed higher expression of osteopontin and osteocalcin mRNA as osteoblastic markers. The expression of MYOD as a myoblast marker was strongly reduced following
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![Graphs and images]

**FIGURE 2. Optimization of odontogenic differentiation of α7⁺ hSMSCs—effect of scaffold.** A, defining culture conditions using specific ECM ligands to support and promote α7⁺ hSMSC differentiation into odontoblast-like cells. The cells were first cultured to form EB-like aggregates and then treated with RA before testing growth on ECM substrates. To evaluate the effect of ECM, the EBs derived from α7⁺ hSMSCs were cultured on ECM substrate for 14 days, and cell proliferation was examined by BrdU cell proliferation ELISA. Error bars represent S.D. (control versus *, p < 0.05; **, p < 0.01). B, proliferation of cells on GS. BrdU cell proliferation ELISA was used to evaluate the proliferation of cells on GS for up to 14 days. α7⁺ hSMSCs were cultured in the absence or presence of the indicated concentration of GS in triplicate wells. Controls were cultured on non-coated culture wells. Data are described as mean ± S.D. and are representative of at least three independent experiments. Error bars represent S.D. Differences between untreated and GS-treated groups were assessed by Mann-Whitney U test (control versus *, p < 0.05; **, p < 0.01). C, expression of differentiation markers during odontoblast differentiation induced in the presence of BMPs. α7⁺ hSMSCs were first cultured to form EB aggregates and then treated with RA followed by culture for 7 days on GS in the absence (control (Cont)) or presence of specific BMPs. Differentiation marker expression was assessed by real-time PCR for MYOD (MYOD1) as muscle marker, DSPP/DMP-1 as odontoblast markers, and osteopontin (SPP1)/osteocalcin (BGLAP) as osteoblast markers. Data are described as mean ± S.D. and are representative of at least three independent experiments. Error bars represent S.D. Differences between untreated and GS-treated groups were assessed by Mann-Whitney U test (control versus *, p < 0.05; **, p < 0.01) as indicated by the brackets. D, expression of lineage markers. Shown are the relative expression levels of MYOD, FOXD3, SOX10, MEOX1, RUNX2, and DSPP in α7⁺ hSMSCs following the differentiation protocol. EBs were formed and exposed to RA for 3 days (days 2–5) and then cultured in GS/BMP-4 for 7 days (days 5–12). Data were obtained by RT-PCR and Western blotting.

differentiation when BMP-4 (100 ng/ml) was used as an odontoblast differentiation factor in the current system. Similar evidence was also obtained with ELISAs for MYOD, DSPP, DMP-1, osteopontin, and osteocalcin (data not shown).

We also followed detailed differentiation patterns expressed during the sequential staged protocol by analysis of messenger RNA and protein expression levels of relevant markers. Markers were estimated in control α7⁺ hSMSCs, compared with cells derived after EB-like aggregate formation with RA, and finally mapped following the GS/BMP-4 differentiation protocol (Fig. 2, C and D). The expression of MYOD mRNA as a marker of myogenic cells was effectively suppressed after treatment with RA, whereas expression of the neural crest markers FOXD3 and SOX10 was clearly up-regulated. For this analysis, we also included the osteogenic transcription factor RUNX2, which is essential for odontoblast differentiation and is a marker of mesenchymal cells with osteoblast/odontoblast potential. Not only was RUNX2 induced, but the expression of mesenchymal MEOX1 was maintained. Next, processing cells under the GS/BMP-4 protocol led to the loss of FOXD3, SOX10, and MEOX1 markers but induced expression of the odontogenic marker DSPP. Therefore, we conclude that the parental α7⁺ hSMSCs retained a myogenic specification that after RA treatment shifted to a neural crest lineage and after the GS/BMP-4 platform differentiated to odontoblast-like specification.

Expression of Odontogenically Related Marker Genes in the Differentiated Cells Derived from α7⁺ hSMSCs—By optimizing a differentiation protocol that includes EB formation and RA treatment followed by BMP-4 growth factor treatment in a permissive culture environment, we were able to induce cells that express markers for odontogenic differentiation. Next, studies examined additional indicators of odontoblastic differentiation following the GS/BMP-4 protocol. As before, the mRNA encoding the transcript for DSPP was induced in differentiated cells, but also mRNA levels for MMP-20 were up-regulated (Fig. 3A). In contrast, expression of MYOD mRNA as a myoblast marker was lost in differentiated α7⁺ hSMSCs on day 12 compared with its expression on day 0 (Fig. 3A). Because osteoblastic markers such as osteopontin and osteocalcin were not expressed in undifferentiated and differentiated α7⁺ hSMSCs (Fig. 2D), we concluded that α7⁺ hSMSCs differentiated into odontoblastic cells but not osteoblastic cells. Although the rat odontoblast-like KN-3 cells showed DSPP and MMP-20, no
differentiation following GS/BMP-4 treatment evaluated by Western blot.

GS/BMP-4 produced a decrease in the α7⁺ hSMSC myoblast marker MYOD but induced odontogenic markers DSPP and MMP-20. A, indicators of odontoblastic differentiation following GS/BMP-4 treatment evaluated by Western blot. α7⁺ hSMSCs were cultured for 7 days with GS/BMP-4 and processed for Western blotting using anti-DSPP, anti-MMP-20, anti-MYOD, and anti-β-tubulin. GS/BMP-4 produced a decrease in the α7⁺ hSMSC myoblast marker MYOD but induced odontogenic markers DSPP and MMP-20. B, indicators of odontoblastic differentiation following GS/BMP-4 treatment evaluated by Western blot. α7⁺ hSMSCs were cultured for 7 days with GS/BMP-4 and processed for Western blotting using anti-DSPP, anti-MMP-20, anti-MYOD, and anti-β-tubulin. GS/BMP-4 produced a decrease in the α7⁺ hSMSC myoblast marker MYOD but induced odontogenic markers DSPP and MMP-20. C, immunofluorescence staining and Western blotting with DSP. α7⁺ hSMSCs show poor staining for DSP in control cells (panel a), but DSP was strongly induced in α7⁺ hSMSCs following odontogenic differentiation (panel b). Scale bar, 50 μm. Panel c, GS/BMP-4 induced odontogenic marker DSP. D, analysis of the expression of osteoblast-related differentiation markers. α7⁺ hSMSCs were cultured using the GS/BMP-4 protocol and processed for real time PCR using primers specific for MYOD (MYOD1), DSPP, MMP-20, osteopontin (SPP1), osteocalcin (BGLAP), and 18S amplicon rRNA. Error bars represent S.D. E, mineralization following induced odontogenic differentiation. Calcium deposition and alkaline phosphatase activities were detected in control α7⁺ hSMSC cultures (panels a, c, and e) and in cultures following the GS/BMP-4 protocol (panels b, d, and f). Cultures were analyzed for morphological changes (panels a and b), ALPase activity (panels c and d), and mineralization (panels e and f) to identify odontogenic lineage. In the absence of the GS/BMP-4 protocol, control cells showed evidence of formed multinucleated myotubes after culture for 7 days (panels a, c, and e, arrows) but were negative for ALPase activity (panel c) and mineralization (panel e). α7⁺ hSMSCs treated with GS/BMP-4 did not exhibit multinucleated cells but showed dendrite-like extensions (panel b) and strong induction of ALPase activity (panel e) and mineralization (panel f). F, analysis of ALPase activity and mineralization following odontogenic differentiation of α7⁺ hSMSCs with the GS/BMP-4 protocol. Panel a, ALPase activity analysis measured at 405 nm and normalized with total protein. Panel b, ARS quantification measured at 405 nm. Groups were assessed by Mann-Whitney U test. Error bars represent S.D. (control versus *, p < 0.01). Cont, control.

Odontoblastic marker was detected in mouse osteoblastic MC3T3-E1 cells (Fig. 3A). Comparable results were also obtained when we performed Western blotting for DSPP, MMP-20, MYOD, and DSP (Figs. 2D and 6B).

To further evaluate the odontogenic potential of α7⁺ hSMSC, DSP immunofluorescence staining was performed. The level of DSP staining in α7⁺ hSMSCs was strongly increased following odontogenic differentiation (Fig. 3C, panels a and b). Taken together with Figs. 2C and 3A, B and C, we confirmed that differentiated cells derived from α7⁺ hSMSCs using the described culture protocol show multiple features characteristic of odontoblastic lineage. The differentiated α7⁺ hSMSCs were observed to have spread outward with dendrite-like extensions from the attached EBs. When α7⁺ hSMSCs were grown under low serum conditions, the effect of BMP-4, a potent inducer of the odontogenic pathway, was tested. They failed to differentiate into myotubes and instead differentiated along the odontogenic pathway (Fig. 3E, panels a and b). It is well known that α7⁺ hSMSCs exhibit multilineage potential similar to induced pluripotent stem cells and embryonic stem (ES) cells, including osteogenic differentiation capacity. Although DMP-1 and MMP-20 are relatively specific markers for odontoblasts, they may not strictly distinguish between odontogenic and osteogenic differentiation. Therefore, to confirm that the cells possess odontogenic differentiation capability, we performed real time PCR for osteoblastic markers in osteoblasts derived from α7⁺ hSMSCs as the comparison experiment (Fig. 3D). The osteoblastic markers osteopontin and osteocalcin were detected in osteoblasts but not in the odontoblast-like cells, which is evidence that the α7⁺ hSMSCs had differentiated along an odontoblastic lineage.

We also investigated the induction of ALPase as an odontoblast marker and found that a majority of GS/BMP-4-treated cells, but not control cells, showed potent expression of the enzyme (Fig. 3E, panels c and d). When we tested whether the GS/BMP-4 protocol induced mineralization in the α7⁺ hSMSCs as evaluated by staining with ARS, we found extensive deposits of matrix in the GS/BMP-4-treated cells but not in control cells (Fig. 3E, panels e and f). Interestingly, as the odontogenic cul-
Our findings provided evidence that the odontogenic lineage is dependent on α1 integrin expression. Furthermore, we demonstrated that the adhesion phenotype of hSMSCs is modulated by changing α1 integrin expression following odontogenic differentiation. Our data suggested that α7 integrin expression is critical for the attachment of hSMSCs to laminin substrates. Moreover, we observed that the myogenic stem cells differentiated in the presence of α7 integrin-blocking mAbs exhibited poor adhesion to collagen substrates. The 9.1 mAb to α7 substantially inhibited cell adhesion to laminin 111 but did not completely abolish it (Fig. 5A). Myoblast adhesion to Col-I was near background levels (Fig. 5B), suggesting that the cells did not express functional receptors for this ligand. Odontogenic differentiation following GS/BMP-4 treatment resulted in a shift in adhesion profile where attachment to laminin 111 substrates was dramatically reduced (Fig. 5A). Control α7+ hSMSCs adhered to laminin with an efficiency of about 60%, whereas only about 20% of the cells were able to attach following GS/BMP-4 treatment. Moreover, for the minor fraction of odontogenic cells that were able to attach, the adherence was sensitive to α1 or β1 integrin-blocking mAb (Fig. 5A). This is consistent with the importance of the α1 integrin in mediating adhesion to laminin 111 in GS/BMP-4-differentiated cells. In contrast, anti-α7 had no apparent effect on adhesion to laminin 111 in these cells. The α7-sorted myoblasts displayed poor adhesion on Col-I substrates, and only about 10% of the cells were able to adhere to this interstitial ligand (Fig. 5B). However, there was a striking increase in adhesion efficiency to Col-I following GS/BMP-4-induced differentiation (Fig. 5B). Analysis with blocking anti-integrin antibodies showed that attachment of odontoblast-like cells to collagen was mediated primarily by the α1 integrin, whereas blocking with a panel of α chain mAbs was without effect (Fig. 5B). As expected, the partner β1 subunit was involved as indicated by the complete block of adhesion with anti-β1 mAb.

We also examined the result of GS/BMP-4-induced differentiation on cell mobility (Fig. 5C). In these studies, parallel assays examined migration on laminin 111 and collagen substrates. On laminin 111, the α7+ hSMSCs showed a strong and persis-
tent migration response (Fig. 5C). Consistent with the high α7 integrin expression, migration was significantly inhibited with the 9.1 anti-α7 mAb. Similarly, anti-β1 antibody was effective in inhibiting motility. Following treatment with GS/BMP-4, motility was reduced by 60% compared with untreated control cells (Fig. 5C). Interestingly, the moderate level of cell migration on laminin by the GS/BMP-4 cells was mediated by α1 integrin as shown by antibody inhibition assays. This inhibitory effect with anti-α1 mAb was similar to that generated by the anti-β1 antibody. Motility was also tested on Col-I substrates (Fig. 5D). α7⁺ hSMSCs typically showed poor migration on this ligand (Fig. 5D). This was expected as they lack high expression of collagen receptors (Fig. 5A). In contrast, cells induced with GS/BMP-4 showed a dramatic increase in migration on collagen (Fig. 5D). This strong response was dependent on the α1β1 receptor because antibodies to either α1 or β1 subunits completely abolished motility. We also confirmed that the addition of anti-α1 integrin antibody to culture for 7 days had no effects on the cell detachment, cell viability, cell death, or cytotoxicity (data not shown).

siRNA Silencing of α1 Integrin Blocks Odontoblastic Differentiation—To examine whether the up-regulation of α1 integrin expression in the cells is an important step during odontoblastic differentiation, target cells were treated using an siRNA knockdown approach. α7⁺ hSMSCs were transfected with α1 integrin siRNA or a control scrambled siRNA and then cultured using the protocol described above. As seen in Fig. 6A, α1 integrin was strongly expressed in differentiated cells transfected with control siRNA, but expression was suppressed in cells transfected with α1 integrin siRNA as shown by RT-PCR and Western blot analysis (Fig. 6A). There was no change in the expression of the GAPDH housekeeping gene and β1 integrin protein levels following siRNA treatment, demonstrating the specificity of the siRNA knockdown. Control siRNA had no effect on α1 integrin mRNA levels or on α1 integrin protein expression. Importantly, we found that transfection of α1 integrin siRNA efficiently prevented induction of the odontoblast differentiation markers DSPP and MMP-20 (Fig. 6B). In addition, following pretreatment with α1 integrin siRNA, the induc-

**FIGURE 5.** Differentiation of α7⁺ hSMSCs with the GS/BMP-4 protocol modulates cell adhesion and motility. Adhesion of α7⁺ hSMSCs (A and B, gray bars) and GS/BMP-4-treated cells (black bars) on laminin 111 (5 μg/ml; A) and Col-I (1 μg/ml; B) was assayed in the absence (control (Cont)) or presence of the indicated anti-integrin antibodies. Data are presented as a percentage of total input cell number. Error bars represent S.D. Migration of control α7⁺ hSMSCs (gray bars) and cells treated with GS/BMP-4 (black bars) on laminin-1 (laminin 111) (5 μg/ml; C) and Col-I (1 μg/ml; D) was assayed in the absence (control) or presence of the indicated anti-integrin antibodies. Cells were added to the upper chamber and incubated for 3 h. Motility was estimated by counting the number of cells that migrated to the undersides of the membranes. The results are averages of at least 10 random microscopic fields. Error bars represent S.D.
tion of ALPase activity was markedly suppressed (Fig. 6, C and E). Similarly, when we tested whether α1 integrin siRNA had an effect on α7⁺ hSMSC mineralization as evaluated by staining with ARS, we found that the siRNA blocked the accumulation of matrix in the GS/BMP-4-treated cells (Fig. 6, D and E). The inclusion of a function-blocking anti-α1 integrin antibody, but not control antibody, also suppressed the expression of odontoblastic markers including DSPP and MMP-20 following differentiation with GS/BMP-4 (data not shown). These results show that the expression of α1 integrin is required for odontoblast-specific functions in α7⁺ hSMSC-derived cells and implicate a role for functional α1 integrin in regulating the differentiation of α7⁺ hSMSCs to odontoblasts.

DISCUSSION

In the current studies, we have established conditions for the efficient conversion of human muscle stem cells to an odontoblast lineage. The protocol is based on the optimization of differentiation inducers and culture conditions and is without the need of epithelial-mesenchymal interaction. The approach for generation of odontoblasts follows a sequential strategy that includes treatment of spheroidal muscle stem cells with RA to yield a neural crest-related cell population followed by expo-
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sure to BMP-4 in a specific scaffold system to generate odontoblast-like cells.

We confirmed that the differentiated skeletal muscle stem cells forced to differentiate with BMP-4 acquired specific functions as evidenced by the appearance of odontoblastic phenotypes including induction of alkaline phosphatase and the synthesis of abundant mineralized extracellular matrix. Although the two-step differentiation protocol described here for muscle stem cells includes BMP-4, which seems effective for generating odontoblasts, earlier work by several groups (26–28) has shown that BMP-2 is important for odontoblast differentiation using dental pulp cells.

After primary treatment of tongue-derived skeletal muscle stem cell aggregates with RA, they exhibited a neural crest phenotype as evidenced by the induction of FOXD3 and SOX10 signals. The expression of RUNX2 in the RA-treated aggregates also substantiates a mesenchymal specification. Interestingly, during embryonic development, the tongue is formed by a complex process where the structure is infiltrated by neural crest precursors along with myoblasts that have migrated from the occipital somites (29–31).

The secondary differentiation step with BMP-4 under gelatin scaffold culture triggered the loss of both FOXD3 and SOX10 neural crest markers with concurrent suppression of RUNX2 expression. Exposure of RA-treated cell aggregates to BMP-4 triggered their odontoblastic differentiation as measured by a coordinate increase in the expression of a specific set of mineralization-regulating genes. Several non-collagenous proteins important in mineralization are expressed in both bone and dentin but at different levels (32–34). The ECM protein DSPP is thought to be more tooth-specific and is strongly expressed by odontoblasts (32, 35). Similarly, DMP-1 protein is expressed at higher levels in teeth (32, 36).

Other protein indicators including alkaline phosphatase, osteocalcin, and osteonectin have been utilized to identify odontoblastic lineage (37, 38). Because both bone and teeth express these markers at variable levels depending on their stage of differentiation, this group of protein indicators has limited use in discriminating between osteoblast and odontoblast lineages. However, MMP-20 (enamelysin) is thought to be highly tooth-specific, and its expression has been found in epithelial ameloblasts and mesenchymal odontoblasts (34, 35). Analysis revealed that both cultured ameloblastic and odontoblastic cells, but not osteoblastic cells, expressed high levels of MMP-20 protein (39). In addition, studies in developing calvariae indicate extremely low to undetectable levels of MMP-20 mRNA (40), suggesting that MMP-20 is a fairly specific marker for odontoblasts. Based on these detailed marker analyses, we conclude that BMP-4 induces abundant odontoblast differentiation from precursor cells produced in the RA-treated EBs derived from human muscle stem cells.

BMP-4-induced differentiation led to the simultaneous up-regulation of the tooth-specific proteins DSPP, DMP-1, and MMP-20, all consistent with a high degree of odontoblast maturation. DSPP is cleaved immediately after secretion into two daughter proteins, i.e. DSP and dentin phosphoprotein (32, 41). DMP-1 also is expressed by differentiating odontoblasts during development (42). The expression of DSPP and DMP-1 in functional odontoblasts in early stages of odontogenesis is consistent with the role that both DSPP and DMP-1 play in the mineralization of dentin (43).

The human muscle stem cells used in these studies are myogenic progenitors committed to muscle lineage, functioning as stem cells that retain capacity for self-renewal; however, these cells also display potential for multipotency. Pure populations of \(a_7^+\) human muscle stem cells were isolated using FACS following labeling with mAb to \(\alpha_7\) integrin. Analysis showed that these isolated cells consistently expressed muscle lineage markers including Myf-5, MyoD, M-cadherin, Pax7, and \(\alpha_7\) integrin (25), were able to undergo myogenesis, and were capable of converting to other mesenchymal differentiation programs.

Although we and others have previously reported osteoblast and adipocyte differentiation from muscle stem cells (14, 44–48), this report is the first to show that using the optimized differentiation protocol \(a_7^+\) hSMSCs were induced to differentiate into odontoblasts and express odontogenesis-related molecules including DSPP and MMP-20. Moreover, in terms of differentiation markers, odontoblasts converted from \(a_7^+\) hSMSCs appear to reach at least a similar level of maturity of differentiating cells compared with those derived from other models.

The present results have identified a novel role for BMP-4 in modulating muscle satellite cell integrin expression and altering their interactions with the microenvironment, leading to odontogenic differentiation. Following the RA/BMP-4 treatment protocol, the muscle stem cells were induced to differentiate along the odontogenic lineage where expression of the \(\alpha_7\) integrin was lost. In parallel, the \(\alpha_1\) integrin was strongly induced and promoted enhanced functions of adhesion and migration on type I collagen substrates. Thus, during early odontogenic differentiation, the drastic coordinate integrin profile switching promoted a dramatic alteration in the cellular response to ECM scaffolding with a newly acquired interaction with type I collagen, the major extracellular matrix protein in dentin. These augmented behavioral functions may facilitate terminal differentiation/maturation, growth, specialized function, and their correct positioning within the tissue microenvironment.

Significantly, the expression of \(\alpha_1\) integrin in cells derived from \(a_7^+\) hSMSCs appeared to be necessary for induction of differentiation into odontoblast-like cells. Specific targeting of \(\alpha_1\) integrin expression using siRNA integrin knockdown strategies during early phase differentiation prevented the final stage of precursor cell conversion to mature odontoblast differentiation. Although little is known about how extracellular signals are able to influence stem cell differentiation, the modulation of expressed adhesion receptors may not only be important during their recruitment to target tissues but may also provide crucial cues for terminal differentiation and tissue-specific regeneration.

Integrins are known to mediate the signaling interface, generating layers of cross-talk between growth factor receptors and ECM scaffold proteins, creating unique and dynamic mechanosensitive microenvironments. This complex boundary forms the stem cell niche responsible for maintenance of stem cell homeostasis and drives lineage commitment and differentiation (49–51). The current findings highlight a mechanism by
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which expression of a specific integrin profile plays a role in establishing and maintaining differentiation status.

The final differentiation of stem cell precursors to an odontogenic lineage depends on the loss of α7 integrin and expression of the α1 integrin. It is not clear how this dependence on α1 regulates differentiation in this pathway, and further work is needed to identify which biochemical and biomechanical signals may be involved in defining the stem cell niche, differentiation potential, and lineage specification.

The detailed mechanisms by which this differentiation pathway is regulated by integrin expression may also depend on the adhesion receptor capacity to sense biophysical cues such as ligand composition, matrix topography, elasticity, and cellular mechanotransduction. Additional studies are planned to define how loss of α1 integrin expression in differentiating odontoblasts induces reversion to hSMSCs. This process appears to involve conversion of one differentiated cell type into another.

Another related, important process is that by which integrins and partner ECM interact to modulate the response to the stem cell niche and regulate the equilibrium between differentiation and stem cell renewal (52, 53). Substantial evidence has accumulated demonstrating the relevance of β1 integrins in regulating stem cell fate. In the local stem cell microenvironment, mechanical forces generated between adherent cells and scaffold ECM have important influences on cell shape, motility, proliferation, and stem cell differentiation status. In particular, elastic microenvironment substrates can determine lineage specification. For example, a flexible scaffold can promote neurogenic lineage commitment by triggering integrin endocytosis, whereas a rigid scaffold favors osteogenic commitment of mesenchymal stem cells (54).

The generation of strategies for organ and tissue regeneration and repair is difficult due to the biological complexity of the three-dimensional structure and diverse tissue architecture along with needed vascularization, innervation, and deposition of extracellular matrix scaffolding. Compared with other organs, the tooth represents an organ of modest size and complexity that comprises an abundance of mineralized extracellular matrix and appears to be feasible for stem cell-based organ regeneration. When teeth are damaged by bacterial infection, traumatic injury, or wear, their repair is difficult without using less functional artificial materials. The potential of stem cells to initiate renewal of dental tissues may yield novel approaches to therapeutic approaches that include generation of odontogenic cells.

Recently, there have been several reports on the odontoblastic transformation of stem cells isolated from adult and neonatal tissues (1, 4, 42). However, the clinical application of these cells has been limited because such stem cells are not abundant, are difficult to isolate and propagate, and have potential for allogeneic rejection. Possible alternative stem cells from other tissues including skeletal muscle could potentially be adapted for tooth organ regeneration, and these new approaches for teeth regeneration need to be explored. Although muscle stem cells are not normally involved in the odontoblast differentiation pathway, these pluripotent stem cells are readily available and could have significant potential for tissue engineering of the human tooth and other tissues. Additional studies are planned to examine the potential of the differentiated muscle-derived precursors to differentiate in vivo by xenograft implantation under the renal capsule.

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