ORIGINAL ARTICLE

Fibroblast Growth Factor Ligand Dependent Proliferation and Chondrogenic Differentiation of Synovium-Derived Stem Cells and Concomitant Adaptation of Wnt/Mitogen-Activated Protein Kinase Signals

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Cell expansion techniques commonly utilize exogenous factors to increase cell proliferation and create a larger cell population for use in cell-based therapies. One strategy for cartilage regenerative therapies is autologous stem cell expansion and fibroblast growth factor (FGF) supplementation during cell expansion, particularly FGF-2. However, it is unknown whether FGF-10, another FGF implicated in limb and skeletal development, can elicit the same rejuvenation responses in terms of proliferation and differentiation of human synovium-derived stem cells (SDSCs). In this study, we expanded SDSCs in either FGF-2 or FGF-10 for 7 days; a control group had no treatment. FGF-2 and FGF-10 supplementation was also exclusively tested during the differentiation phase. Expanded SDSCs were evaluated for their ability to successfully engage in chondrogenic and osteogenic differentiation. We found that FGF-2 supplementation during proliferation, but not differentiation, was able to increase glycosaminoglycan deposition, pellet size, and chondrogenic gene expression following chondrogenic induction, as well as increased calcium deposition, alkaline phosphatase activity, and expression of vital osteogenic differentiation genes following osteogenic induction. FGF-10 did not elicit a similar pre-conditioning effect. We also observed changes of both Wnt signals and mitogen-activated protein kinase expression during SDSC chondrogenesis, which occurred in a manner dependent upon the supplementation phase of FGF-2 administration. These results indicated that FGF-2, but not FGF-10, may be supplemented during stem cell expansion to prime cells for successful chondrogenesis and osteogenesis.

Introduction

ADULT MESENCHYMAL STEM CELLS (MSCs) are promising alternative cell sources for cartilage tissue engineering due to the shortage of autologous chondrocytes for cell-based regenerative therapies.¹ However, adult stem cells obtained from tissues have either less chondrogenic potential despite large amounts such as adipose stem cells or higher endochondral ossification and limited sample size such as bone marrow stromal cells (BMSCs).² One promising MSC population found in the joint, known as synovium-derived stem cells (SDSCs), has recently been characterized as tissue-specific stem cells for chondrogenesis.³ Strategies which allow for MSC proliferation may be necessary to achieve a substantial and usable cell number for therapies;

however, the initial MSC numbers are low and their differentiation potential can be compromised following excessive *ex vivo* expansion, with notable increases in cell senescence marker expression and decreased proliferative capacity.⁴ This situation can be further complicated when combined with other preexisting and potentially detrimental factors such as donor age and disease pathology.⁵

It is known that the fibroblast growth factor (FGF) family is involved in limb and joint development, as well as various stages of skeletal and cartilage formation and maturation.⁶ For instance, FGF-2 is involved in early cartilage development and can cause dramatic increases in cell proliferation in chondrocytes and osteoblasts.⁷ In addition, another member of the FGF family, FGF-10, is known to be vital to limb bud initiation and development,^{8,9} but less is known about the

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FIG. 1. Gene expression of FGF ligands in cell expansion phase and chondrogenic markers in chondrogenic induction phase following the dECM pretreatment. The study design was detailed previously.¹⁸ Briefly, human SDSCs were expanded on either dECM or Plastic for one passage followed by a 2-week chondrogenic induction. Microarray analysis was used to evaluate FGF ligand genes in expanded cells (A) and chondrogenic marker genes in differentiated cells (B). The raw data were uploaded into Partek (St. Louis, MO) software for initial analysis. After raw intensity was background-subtracted, robust multiarray analysis was normalized, log transformed, and fold changes were determined. Heatmap.2 in R was used to show the effects on FGF ligand and chondrogenic genes, annotated in Ingenuity Pathway Analysis (IPA, Redwood City, CA) as affecting cell proliferation and chondrogenic differentiation, respectively. dECM expanded cells were referred to as Ec, while Plastic expanded cells were referred to as Pc (A). The pellets from dECM expanded cells were referred to as Ep, while those from Plastic expanded cells were referred to as Pp (B). FGF, fibroblast growth factor; dECM, decellularized extracellular matrix; SDSC, synovium-derived stem cell. Color images available online at www.liebertpub.com/tea

ability of FGF-10 to modulate chondrogenic activity, although a recent report indicated that FGF-10 promoted Meckel's cartilage regeneration in rats.¹⁰ Interestingly, FGF signaling has been shown to maintain MSCs in an undifferentiated state during proliferation while preserving their multipotentiality,¹¹ which further establishes the FGF family's potential benefits for preconditioning strategies.

Our recent findings suggest that decellularized extracellular matrix (dECM) deposited by stem cells could also provide a

preconditioning strategy on which stem cells could be greatly expanded with enhanced chondrogenic potential¹² or endochondral ossification.¹³ Interestingly, microarray data from these dECM studies have shown that, among all FGFs, FGF-2 and FGF-10 are the most significantly regulated factors in human SDSCs following their expansion on dECM (Fig. 1). It is unknown whether a similar rejuvenating effect can be achieved on human SDSC chondrogenesis and osteogenesis through FGF-2 and FGF-10 supplementation in the cell expansion phase, as well as supplementation in the differentiation phase. Due to the importance and potential impacts in stem cell-mediated chondrogenesis,^{14,15} the content of both Wnt and mitogen-activated protein kinase (MAPK) activation, which have been demonstrated in our earlier dECM preconditioning study,¹⁶ will also be assessed in this study following FGF ligand rejuvenation.

Materials and Methods

Evaluation of cell proliferation, surface phenotypes, and differentiation genes

Cell culture and proliferation. Human adult SDSCs obtained from Asterand (North America Laboratories, Detroit, MI) were cultured in growth medium containing alpha-minimum essential medium, 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone as described previously.^{16–18} Passage 4 SDSCs were treated with 10 ng/mL of FGF-2 or FGF-10 (Pepro-Tech, Inc., Rocky Hill, NJ) during cell expansion/proliferation (F2P and F10P, respectively) or differentiation (F2D and F10D, respectively) (Fig. 2A). Cells cultured with no FGF treatment acted as a control (CNTL). Cell number was counted in 175 cm² flasks ($n=6$) using a hemocytometer. To determine proliferation index, before cell expansion, SDSCs were labeled with CellVue® Claret at 2×10^{-6} M for 5 min according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). After 6 days of proliferation, expanded cells were collected and measured using a BD FACSCalibur™ flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences), and cell proliferation index was analyzed by ModFit LT™ version 3.1 (Verity Software House, Topsham, ME).

Surface phenotypes of expanded cells. The following primary antibodies were used in flow cytometry analysis to detect expanded SDSC surface immunophenotype profiles: CD29 (Abcam, Cambridge, MA), CD90 (BD Pharmingen, San Jose, CA), CD105 (BioLegend, San Diego, CA), the stage-specific embryonic antigen 4 (SSEA4; BioLegend), and isotype-matched immunoglobulin Gs (IgGs; Beckman Coulter, Fullerton, CA). The secondary antibody was goat anti-mouse IgG (H + L) R-phycoerythrin conjugated (Life Technologies, Carlsbad, CA). Samples ($n=3$) of each 2×10^5 expanded cells were incubated on ice in cold phosphate-buffered saline containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN₃ (Sigma-Aldrich) for 30 min. The cells were then sequentially incubated in the dark in the primary and secondary antibodies for 30 min. Fluorescence was analyzed by a FACSCalibur (BD Biosciences) using FCS Express 4 software package (De Novo Software, Los Angeles, CA).

FIG. 2. FGF ligand mediated human SDSC proliferation. (A) Experimental design. Five groups in total: FGF-2 and FGF-10 were only included in a 7-day cell expansion phase (F2P and F10P, respectively) or in a 21-day differentiation phase (F2D and F10D, respectively) with non-FGF treatment as a control (CNTL). (B) Flow cytometry was used to measure proliferation (prolif.) index of expanded SDSCs. (C) Cell number was counted ($\times 10^6$) after a 7-day cell expansion on 175 cm^2 flasks ($n=6$). (D). Flow cytometry was used to measure both percentage and median fluorescence intensity of mesenchymal stem cell surface markers (CD29, CD90, CD105, and SSEA4) of expanded SDSCs. SSEA4, stage-specific embryonic antigen 4. Color images available online at www.liebertpub.com/tea

Senescence and differentiation gene expression. Expanded cells were evaluated using real-time polymerase chain reaction (PCR) for senescence and differentiation-related gene changes. Total RNA was extracted from representative samples ($n=4$) using an RNase-free pestle in TRIzol[®] (Life Technologies). Two micrograms of mRNA were used for reverse transcriptase with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) at 37°C for 120 min. Senescence-related genes (cyclin-dependent kinase inhibitor 2A [*P16*; Assay ID Hs00923894_m1], cyclin-dependent kinase inhibitor 1A [*P21*; Assay ID Hs00355782_m1], and tumor protein p53 [*TP53*; Assay ID Hs01034249_m1]), chondrogenic marker genes (SRY [sex determining region Y]-box 9 [*SOX9*; Assay ID Hs00165814_m1], aggrecan [*ACAN*; Assay ID AIQJAP5], and type II collagen [*COL2A1*; Assay ID Hs00156568_m1]), and osteogenic genes (Runt-related transcription factor 2 [*RUNX2*; Assay ID Hs00231692_m1]; Osterix [*SP7*; Assay ID Hs01866874_s1]; Secreted Phosphoprotein 1 [*SPP1*; Assay ID Hs00959010_m1]; Bone sialoprotein [*IBSP*; Assay ID Hs00173720_m1]; and Bone Gamma-Carboxyglutamate [Gla] Protein [*BGLAP*; Assay ID Hs01587814_g1]) were customized by Applied Biosystems as part of their Custom TaqMan[®] Gene Expression Assays. Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*; Assay ID Hs02758991_g1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ[™] Multicolor RT-PCR Detection and calculated by computer software (PerkinElmer, Wellesley, MA). Relative transcript levels were calculated as $\chi = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta E - \Delta C$, $\Delta E = C_{t_{exp}} - C_{t_{GAPDH}}$, and $\Delta C = C_{t1} - C_{t_{GAPDH}}$.

Chondrogenic induction and evaluation

Chondrogenic induction. Expanded cells (3.0×10^5) were centrifuged at 500 *g* for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation (day 0), the pellets were cultured in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's modified Eagle's medium, 40 μ g/mL proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1 $\times$ ITS[™] Premix (BD Biosciences) with the supplementation of 10 ng/mL transforming growth factor beta3 (TGF- β 3, PeproTech, Inc.) in a 5% O₂ incubator as long as 21 days. F2D and F10D groups were also supplemented with 10 ng/mL FGF-2 or FGF-10, respectively. The pellets were evaluated using real-time PCR for chondrogenic marker genes (*ACAN* and *COL2A1*), type I collagen (*COL1A1*; Assay ID Hs00164004_m1), hypertrophic genes (type X collagen [*COLXA1*; Assay ID Hs00166657_m1] and alkaline phosphatase [*ALP*; Assay ID Hs01029144_m1]), histology and immunohistochemistry (IHC) for staining, and biochemical analysis for both DNA and glycosaminoglycan (GAG) amounts.

Histology and immunostaining. Representative pellets ($n=2$) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene, and embedding in paraffin blocks. Five-micrometer thick sections were stained with Alcian blue (counterstained with fast red) for sulfated GAGs. For IHC, the sections were immunolabeled with primary antibody

against type II collagen (Col2; II-II6B3, Developmental Studies Hybridoma Bank, Iowa City, IA), followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using VECTASTAIN ABC reagent (Vector) with 3, 3'-diaminobenzidine as a substrate.

Biochemical analysis for DNA and GAG contents. Representative pellets ($n=4$) were digested at 60°C for 4 h with 125 μ g/mL papain in PBE buffer (100 mM phosphate, 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine. To quantify cell density, the amount of DNA in the papain digestion was measured using the Quanti-iT[™] PicoGreen[®] dsDNA Assay Kit (Life Technologies) with a CytoFluor[®] Series 4000 (Applied Biosystems). GAG was measured using dimethylmethylene blue dye and a Spectronic[™] BioMate[™] 3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) with bovine chondroitin sulfate (Sigma-Aldrich) as a standard.

Expression of both Wnt and MAPK signals following FGF-mediated chondrogenesis

Expanded cells and subsequent pellets were dissolved in the lysis buffer (Cell Signaling, Danvers, MA) with protease inhibitors. Total proteins were quantified using BCA[™] Protein Assay Kit (Thermo Fisher Scientific). Thirty micrograms of protein from each sample were denatured and separated using NuPAGE[®] Novex[®] Bis-Tris Mini Gels in the XCell SureLock[™] Mini-Cell (Life Technologies) at 120 V at 4°C for 3 h. Bands were transferred onto a nitrocellulose membrane using an XCell II[™] Blot module (Life Technologies) at 15 V at 4°C overnight. The membrane was incubated with primary monoclonal antibodies in 5% bovine serum albumin in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% TWEEN 20) for 1 h (β -actin served as an internal control), followed by the secondary antibody of horseradish peroxidase-conjugated goat anti-mouse (Thermo Fisher Scientific) for 1 h. SuperSignal West Femto Maximum Sensitivity Substrate and CL-XPosure Film (Thermo Fisher Scientific) were used for exposure. The primary antibodies used in immunoblotting included the MAPK Family Antibody Sampler Kit (extracellular signal-regulated protein kinases 1 and 2 [Erk1/2], Jun N-terminal kinase [Jnk], and p38), phosphorylated (p-) MAPK Family Antibody Sampler Kit, and Wnt Signaling Antibody Sampler Kit (Cell Signaling). Wnt11 polyclonal antibody was obtained from Thermo Fisher Scientific. Wnt signals were also evaluated using real-time PCR (*WNT3A*; Assay ID Hs00263977_m1, *WNT5A*; Assay ID Hs00998537_m1, and *WNT11*; Assay ID Hs00182986_m1) following cell expansion and chondrogenic differentiation.

Osteogenic induction and evaluation

Expanded cells ($n=3$) cultured for 21 days in osteogenic medium (growth medium supplemented with 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate, 50 μ M ascorbate-2-phosphate, and 0.01 μ M 1,25-dihydroxyvitamin D3) were collected for ALP activity assay with a reagent kit (Sigma-Aldrich) by measuring the formation of *p*-Nitrophenol from *p*-nitrophenyl phosphate following the manufacturer's instructions. For evaluation of calcium deposition, induced

cells ($n=3$) were fixed with 70% ice-cold ethanol for 1 h and then incubated in 40 mM Alizarin Red S (ARS) at pH 4.2 for 20 min with agitation. After rinsing, matrix mineral-bound staining was photographed. Quantification of staining was performed by staining density using ImageJ software.

Statistical analyses

Analysis of variance with pairwise comparison and *t*-test was used to compare measurements between different groups. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL). *p*-Values <0.05 were considered statistically significant.

Results

Addition of FGF-2 but not FGF-10 during the expansion phase promoted SDSC proliferation

After 7 days of monolayer expansion, SDSCs without FGF treatment exhibited enlarged cell morphology which, in the presence of FGF-2, became notably smaller and fibroblast-like shaped cells with a glistening outline. Both proliferation index (Fig. 2B) and cell number counting data (Fig. 2C) suggested that FGF-2 significantly enhanced SDSC proliferation, while FGF-10 supplementation did not induce greater cell proliferation compared to the control group. This result is also supported by SSEA4 expression levels evaluated by flow cytometry (Fig. 2D). Surprisingly, other stem cell markers (Fig. 2D), including CD29, CD90, and CD105, exhibited decreases in median fluorescence intensity when SDSCs were expanded in the presence of FGF-2. Similar to the cell proliferation data, FGF-10 treatment did not elicit any meaningful differences in the expression of stem cell markers.

Addition of FGF-2 led to changes in expression of senescence and differentiation-related genes

TaqMan[®] real-time PCR data showed that FGF-2 treatment upregulated *P16* expression level in expanded cells compared with the control group but downregulated senescent genes *P21* and *TP53* expression levels (Fig. 3A). We also found that FGF-2 treatment significantly increased transcriptional factor *SOX9*, and FGF-10 treatment decreased *SOX9* expression. This result was also accompanied by early and dramatic decreases in chondrogenic marker gene expression (*ACAN* and *COL2A1*) (Fig. 3B). For osteogenic genes, FGF-10 preconditioning significantly decreased *RUNX2* expression, while FGF-2 produced similar *RUNX2* expression levels as the control. Interestingly, FGF-2 supplementation, but not FGF-10, led to significant increases in *SP7*, *SPP1*, *IBSP*, and *BGLAP* gene expression versus other groups (Fig. 3C).

Addition of FGF-2 but not FGF-10 during the expansion phase promoted SDSC chondrogenic potential

After 21-day chondrogenic induction, FGF-2 pretreated SDSCs yielded pellets with the largest size and most intense staining for sulfated GAGs by Alcian blue and Col2 by IHC compared to the other four groups, which exhibited similar

FIG. 3. FGF ligand mediated senescence and differentiation-related gene expression in expanded SDSCs. TaqMan[®] real-time PCR was used to quantify mRNA levels of senescent genes (A): *P16*, *P21*, and *TP53*, chondrogenic genes (B): *SOX9*, *ACAN*, and *COL2A1*, and osteogenic genes (C): *RUNX2*, *SP7*, *SPP1*, *IBSP*, and *BGLAP*. Data are shown as average ± SD for $n=4$. Groups not connected by the same letter are significantly different ($p < 0.05$). PCR, polymerase chain reaction; SD, standard deviation; *SOX9*, SRY (sex determining region Y)-box 9; *ACAN*, aggrecan; *COL2A1*, type II collagen; *RUNX2*, Runt-related transcription factor 2; *SP7*, Osterix; *SPP1*, secreted phosphoprotein 1; *IBSP*, bone sialoprotein; *BGLAP*, bone gamma-carboxyglutamate (Gla) protein.

pellet sizes and staining intensities (Fig. 4A). These findings were confirmed by biochemical analysis data in which the FGF-2 pretreatment group yielded the highest DNA ratio by day 0, GAG amount per pellet, and ratio of GAG to DNA, known as the chondrogenic index (Fig. 4B). TaqMan real-time PCR data showed that, after 21-day chondrogenic induction, FGF-2 pretreated cells yielded the lowest expression of *COL1A1*, but the highest levels of *COL2A1* and *ACAN*, while FGF-2 treated cell pellets yielded the second lowest level of *COL1A1* and the lowest levels of *COL2A1* and *ACAN*. Both FGF-10 treatment groups, regardless of supplementation phase, yielded similar expression of *COL1A1* and *ACAN*, which was lower than the control group (Fig. 4C), but still significantly greater than either FGF-2 treatment group. We also found that FGF-2 pretreatment yielded cells with the highest level of *COL10A1* and *ALP*, while FGF-2 treatment yielded cells with the lowest level of *ALP* mRNA (Fig. 4D).

FIG. 4. FGF ligand mediated SDSC chondrogenic differentiation. After a 21-day chondrogenic induction, SDSC pellets were evaluated using Alcian blue staining for sulfated GAGs and immunohistochemical staining for Col2 (**A**), biochemical analysis for both DNA and GAG amounts in a pellet (**B**), and TaqMan real-time PCR for mRNA levels of chondrogenic genes (**C**): *COL1A1*, *COL2A1*, and *ACAN* and hypertrophic genes (**D**): *COL10A1* and *ALP*. Data are shown as average $\pm$ SD for $n=4$. Groups not connected by the same letter are significantly different ($p<0.05$). GAG, glycosaminoglycan; Col2, type II collagen; ALP, alkaline phosphatase. Color images available online at www.liebertpub.com/tea

Wnt and MAPK signals following FGF-2 preconditioning and chondrogenic differentiation

TaqMan real-time PCR data showed that FGF-2 pretreated SDSCs displayed a significant upregulation of *WNT5A* and *WNT3A* and downregulation of *WNT11* (Fig. 5A), which was confirmed by Western blot data (Fig. 5B); compared to significant upregulation of both p-p38 and p-Jnk signals in FGF-2 pretreated SDSCs, total Erk1/2 was markedly reduced with a modest suppression in p-Erk (Fig. 5C). Following chondrogenic differentiation, FGF-2 pretreated SDSCs showed decreased levels of Wnt5a, but increased levels of Wnt11;

however, FGF-2 treatment in chondrogenic induction exhibited an opposite trend (Fig. 5D, E). Interestingly, FGF-2 pretreated SDSCs also showed decreased levels of p38, Jnk, and Erk1/2 compared to other groups (Fig. 5F).

Effect of FGF-2 and FGF-10 on SDSC osteogenic differentiation

After 21-day osteogenic induction, FGF-2 pretreated SDSCs exhibited the highest density of both ARS (Fig. 6A) and ALP staining (Fig. 6B), indicating stronger calcium

FIG. 5. FGF ligand mediated Wnt and MAPK signal changes in SDSC chondrogenesis. During cell expansion (A–C) and chondrogenic induction (D–F), Wnt signals were evaluated using both TaqMan real-time PCR (A, D) and Western blot (B, E), while MAPK signals were evaluated using Western blot (C, F). GAPDH was used as an internal control. Data are shown as average $\pm$ SD for $n=4$. Groups not connected by the same letter are significantly different ($p<0.05$). MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

deposition and ALP expression, respectively. Interestingly, FGF-2 treatment in osteogenic induction significantly decreased ARS staining compared to other groups, while FGF-10 treatment either during cell expansion or osteogenic induction had no evident effect on osteogenic differentiation of expanded SDSCs (Fig. 6C, D).

Discussion

In this study, we sought to determine whether FGF-2 or FGF-10 supplementation during the proliferation phase or the differentiation phase could improve human SDSC chondrogenesis and osteogenesis. In addition, we wanted to determine if both Wnt and MAPK signals were involved in FGF ligand-mediated SDSC proliferation and lineage differentia-

tion. The present study suggested that FGF-2 supplementation during the proliferation phase can precondition SDSCs to undergo more successful chondrogenesis and osteogenesis following differentiation induction, which was superior to FGF-10 supplementation, no treatment, and the addition of either FGF during differentiation. The early upregulation of Wnt3a and Wnt5a and early downregulation of Wnt11 are likely to be influential mechanisms involved in FGF preconditioning and successful differentiation along the chondrogenic and osteogenic lineages. Chondrogenic differentiation was complemented by an upregulation of *SOX9*, a known transcription factor for chondrogenic activity, with FGF-2 supplementation during proliferation. Interestingly, FGF-2 preconditioning during the proliferation phase led to a *RUNX2*-independent mRNA upregulation of several vital osteogenic genes and more

FIG. 6. FGF ligand mediated SDSC osteogenic differentiation. After a 21-day osteogenic induction, both ARS (A) and ALP staining (C) were used to evaluate osteogenic differentiation. ImageJ software was used to semiquantify the density of staining (B, D, respectively). Data are shown as average $\pm$ SD for $n = 4$. Groups not connected by the same letter are significantly different ($p < 0.05$). ARS, Alizarin Red S. Color images available online at www.liebertpub.com/tea

successful osteogenesis *in vitro* than all other groups. These findings suggested that FGF-2 supplementation during the early proliferation phase can prime human SDSCs for successful chondrogenic and osteogenic differentiation, which may be, in part, modulated through Wnt and MAPK signaling, but not when supplemented with FGF-2 during differentiation or with FGF-10 at any stage.

Unlike human germ cells, most human somatic cells do not express telomerase and, therefore, lose telomeric DNA during each round of DNA replication.¹⁹ Stress conditions could cause cell senescence²⁰ resulting from DNA damage triggered by telomere shortening through the p53-mediated signaling pathway; on the contrary, p53 inactivation prolongs the lifespan of human fibroblasts.²¹ The expression of p21, a p53 target gene, increases in senescent cells.²² Since targeted deletion of the p21 gene is sufficient to escape senescence in human fibroblasts,²³ p21 may have a major role in the induction of cellular senescence in human fibroblasts by inhibiting the activity of cyclin-dependent kinases. In this study, preconditioning using FGF-2 dra-

matically decreased *P21* and *TP53*, indicating a rescue of telomere-dependent intrinsic senescence. Interestingly, both FGF-2 and FGF-10 pretreatment dramatically increased *P16* expression in expanded cells, which is usually raised through extrinsic senescence such as reactive oxygen,¹⁹ suggesting that the rescue dominated by FGF-2 pretreatment was independent of the p16/pRb pathway. Future studies will be necessary to fully validate these trends, determine whether these changes are consistent over several cell passages, and discover a possible mechanism in which FGF-2 may be regulating cell senescence.

In this study, we found that FGF-2 rather than FGF-10 promoted SDSC proliferation. The potential underlying mechanisms could be selection of a particular subset of cells by telomere length in FGF-2-expanded MSCs.^{24,25} Despite the role in regulating the proliferation and maintenance of dental epithelial stem cells,²⁶ in this study, FGF-10 did not act as a proliferation promoter like FGF-2. Interestingly, FGF-2 pretreatment decreased CD29, CD90, and CD105 expression, but increased SSEA4. This finding is consistent with a report by Hagmann *et al.* in which they found that

CD90, CD105, and CD146 were significantly decreased in human BMSCs after treatment with FGF-2.²⁷ One potential explanation is that FGF-2 could decrease the expression of TGF- β , which is a promoter of cell senescence.²⁸ Sacchetti *et al.* found that FGF-2 pretreatment decreased the expression of CD105 (endoglin), a TGF- β coreceptor.²⁹ Furthermore, Ito *et al.* found that downregulation of TGF- β signaling was responsible for the rejuvenating effect of FGF-2 pretreatment in terms of osteogenic and chondrogenic potential in human MSCs.³⁰ A similar trend in surface phenotypes was observed in human SDSCs after expansion on dECM deposited by stem cells.^{17,18} Like pretreatment with FGF-2, this dECM expansion approach also plays a role in the preconditioning of expanded stem cells in both proliferation and differentiation capacity.³¹ Since CD29, CD90, and CD105 are considered traditional surface markers for stem cells,³² the decrease in these markers during stem cell rejuvenation raises questions regarding their defined roles as they pertain to stem cell "stemness."

Despite the fact that both chondrogenic and osteogenic potentials were significantly increased after FGF-2 pretreatment, we did find a discrepancy in the expression of transcriptional and differentiation genes for a specific lineage in expanded cells. In terms of osteogenesis, FGF-2 pretreated SDSCs exhibited an upregulation of both transcriptional gene (*SP7*) and matrix genes (*SPPI*, *IBSP*, and *BGLAP*), despite no significant change in another transcriptional gene (*RUNX2*). This finding might be explained by negative feedback modulation through addition of exogenous FGF-2, which can lower endogenous FGF-2 expression,³³ resulting in a decrease of the osteogenesis inhibitory effect exerted by FGF-2 during subsequent osteogenic induction.^{34,35} In terms of chondrogenesis, however, FGF-2 pretreated cells exhibited an upregulation of a transcriptional gene (*SOX9*), but downregulation of matrix genes (*ACAN* and *COL2A1*). This finding could be explained by a recent report in which pretreatment with FGF-2 could significantly enhance reprogramming efficiency by downregulating matrix genes such as collagen.³⁶

Due to the importance of both Wnt and MAPK signaling in cartilage regeneration,¹⁵ the signals associated with these two pathways were characterized during FGF-2 pretreatment and subsequent chondrogenic induction. Following FGF-2 supplementation during the proliferative phase, we found that *WNT3A* and *WNT5A* mRNAs were markedly increased, while *WNT11* mRNA was decreased. Interestingly, a contrasting trend, with *WNT5A* mRNA expression significantly decreased and *WNT11* mRNA significantly increased versus other groups, occurred following 21-day pellet differentiation culture. This result appears to support the notion that Wnt signals are stage-specifically expressed during chondrogenesis.³⁷ For instance, Wnt5a has been previously implicated as an early promoter³⁸ and Wnt11, which has been shown to stimulate Col2 deposition,³⁹ is a late promoter of chondrogenesis.⁴⁰ Another study reported that Wnt3a-canonical and noncanonical Wnt pathways counteracted one another in MSC chondrogenesis.⁴¹ This notion seems well supported by other studies, which suggest that Wnt3a can prevent or inhibit chondrogenesis, while Wnt5a supports cartilage formation, but can impede Col2 expression and induce dedifferentiation.^{39,42,43} These findings support the Wnt expression trends observed in our data, where both Wnt3a and Wnt5a were expressed in earlier

stages following proliferation and FGF-2 supplementation but, following chondrogenic differentiation, Wnt3a and Wnt5a mRNAs and proteins were either significantly decreased or undetected. This expression is likely important in maintaining the "stemness" of the SDSCs, which may promote successful responses to chondrogenic media, as well as later expression of Col2, aggrecan, and greater GAG deposition. In addition, it is worth noting that preconditioning methods, which directly or indirectly regulate Wnt signaling, could hold great potential; however, a study by Hoang⁴⁴ raises a valid point that therapies targeting Wnt may have significant side effects due to their defined roles in tissue regeneration and stem cell self-renewal, as well as osteosarcoma. Although aberrant Wnt signaling does not seem to be a concern in this study, specifically with FGF-2 treatment of SDSCs, as evidenced by the reversed trend in the Wnt real-time PCR results from the expansion phase to the differentiation phase, it is a caveat which should be noted when considering therapies which modify Wnt signaling.

Interestingly, *RUNX2* was not significantly increased in either of the FGF supplemented groups versus the control in expanded cells before osteogenic induction. Furthermore, Western blotting revealed that expanded cells treated with FGF-2 during proliferation had robust levels of p-p38 and p-Jnk, but little p-Erk1/2 or Erk1/2. It is known that *RUNX2*, a transcriptional regulator of osteogenesis, is heavily regulated through the Erk signaling pathway^{45,46}; however, it may not be required for osteogenic differentiation to occur. A study using rat BMSCs reported results demonstrating that inhibition of p38 activity alone did not interfere with osteogenesis and suggested that mechanical strain-induced osteogenic differentiation was a result of Erk1/2 and *RUNX2* activation.⁴⁷ Additional studies have reported that the inhibition of Erk1/2 and Jnk, but not p38, has resulted in inhibited osteogenic differentiation⁴⁸; also, decreased *RUNX2* expression was observed in BMSCs with the inhibition of Erk1/2 phosphorylation.⁴⁶ Furthermore, it has been confirmed that *RUNX2* activation occurs through the Erk pathway.⁴⁵ Although p38 is undeniably implicated in osteogenesis, studies targeting the Erk and Jnk pathways through inhibition seem to suggest that the Erk pathway may be more closely related to *RUNX2* activation and regulation. Given the growing number of studies reporting *RUNX2* activation without p38 signaling, the lack of *RUNX2* expression could very likely be directly related to Erk1/2 signaling, which was significantly decreased in our current study compared to robust levels of p-p38 and p-Jnk in the FGF-2 pretreatment group following cell expansion, as evidenced by Western blotting. It is very possible that increases in ALP and ARS staining following successful osteogenic induction in the FGF-2 pretreatment group are the result of increased gene expression of other key osteogenic genes such as *SP7*, *SPPI*, *IBSP*, and *BGLAP*, which were all significantly increased in the FGF-2 pretreatment group versus control and FGF-10 pretreatment groups, but not through mechanisms related to *RUNX2* or Erk1/2 activation. Overall, it seems that the strong ALP activity, calcium deposition, and upregulation of several key osteogenic genes in the FGF-2 pretreatment group were primarily driven through *RUNX2*-independent and Erk1/2-independent signaling and likely occurred through p38, Jnk, and potentially other signaling mechanisms. More studies need to be performed to fully assess the necessity of Erk1/2 and *RUNX2* roles in SDSC osteogenesis.

In conclusion, FGF-2 preconditioning led to superior cell proliferation, chondrogenesis, osteogenesis, and overall rejuvenation versus no treatment or FGF-10 preconditioning. Cells preconditioned with FGF-2 during the proliferative phase led to robust chondrogenic pellet formation, accompanied by significant GAG deposition and upregulation of vital chondrogenic gene expression. Likewise, FGF-2 preconditioning produced unmatched osteogenesis, leading to cells with increased gene expression following proliferation, and led to significant increases in ARS and ALP staining for the differentiated cell cultures versus other groups. FGF-2, but not FGF-10, preconditioning was able to elicit significant changes in Wnt signal expression, as well as pronounced p38 signaling. FGF-2 seems to be an optimal choice as a preconditioning stimulus for human SDSC chondrogenesis and osteogenesis.

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Disclosure Statement

No competing financial interests exist.

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