Secreted frizzled-related proteins (SFRPs) are soluble proteins that have highly restricted tissue distribution. Although not fully understood, a role of SFRP1 in the regulation of apoptosis has been suggested. Our previous study disclosed a much greater level of SFRP1 expression in periodontal ligament fibroblasts (PDLFs), which have been suggested to maintain a reduced level of apoptosis compared with gingival fibroblasts. We have tested the role of SFRP1 in the regulation of fibroblast apoptosis both in vitro and in vivo. Our data showed that SFRP1 was significantly up-regulated in cultured human PDLFs during ceramide-induced apoptosis. In vivo study demonstrated an increased SFRP1 expression in mice periodontal ligament during force-induced apoptosis. While inhibition of endogenous SFRP1 expression decreased the percentage of cell death in cultured human PDLFs, exogenous SFRP1 substantially reduced apoptosis in cultured human gingival fibroblasts, which do not maintain a high level of endogenous SFRP1 expression. The effect of SFRP1 on apoptosis was linked to the regulation of several apoptosis-related genes, including p53, caspase-3, caspase-9, and BCL-2-interacting killer (BIK). Furthermore our results indicated that the addition of exogenous SFRP1 could reduce the level of apoptosis in dermal fibroblasts in vitro, and this effect was also linked to the regulation of similar apoptosis-related genes as observed in in vitro studies. Collectively our results suggest that the constitutive up-regulation of SFRP1 could be an adaptive cell survival mechanism inherent to functionally specialized fibroblasts, and the addition of SFRP1 may contribute to the inhibition of apoptosis in fibroblast-related cells.

Apoptosis is a genetically regulated, morphologically distinct form of cell death that plays a major role during development, in homeostasis, and in many diseases (1, 2). The machinery to carry out apoptosis involves various apoptotic components including the BCL-2 gene family and the caspase gene family (3–6). Studies suggest that apoptosis is regulated by diverse functional pathways, each utilizing a subset of apoptotic genes that are active in different cell types (7, 8).

Secreted frizzled-related proteins (SFRPs) are soluble proteins that have a highly restricted tissue distribution (9–11). Studies have indicated that SFRPs are capable of binding to Wnts and frizzled (Fz) receptors to interfere with Wnt signaling, which plays a major role in cell fate determination through the regulation of cell proliferation, differentiation, and apoptosis (12). Distinctive members of the SFRP family exhibit different expression patterns in vivo, suggesting their ability to modulate diverse aspects of Wnt signaling (13–15). Although the details are far from clear, a prominent role for SFRP1 in the regulation of apoptosis has been suggested (16, 17). It has been shown that SFRP1 promotes survival of glioma cells under serum starvation (17) and contributes to the development of uterine leiomyomas through an antiapoptotic effect (18). It was also suggested that SFRP1 may have proangiogenic effects and appears to protect human umbilical vein endothelial cells from apoptosis in vitro (19). Furthermore expression of SFRP1 has been shown to cause spontaneous adipocyte differentiation (20), which is known to render adipocytes resistant to apoptotic stimuli (21). However, there are also studies suggesting a proapoptotic effect of SFRP1 in breast adenocarcinoma cells (16) and in pulmonary epithelial and endothelial cells (22). This leaves the molecular mechanism responsible for the modulation of Wnt signaling and regulation of apoptosis by SFRP1 open to speculation. Indeed SFRP1 has been shown to be a bifocal modulator of Wnt-induced effects at different concentrations (10).

The tooth-supporting structure called periodontium is an excellent model to study fibroblastic cells given that all stages of fibroblast differentiation can be observed in close vicinity. These structures comprise a periodontal ligament responsible for tooth anchoring, tissue regeneration, and homeostasis (23, 24) and gingival tissues responsible for mounting the appropriate inflammatory process in the event of microbial challenge (25, 26). Our recent studies of fibroblast apoptosis in periodontal ligament and gingival tissues indicate that the periodontium is an excellent model system to study the mechanistic control of apoptosis in fibroblastic cells (27). A similar model has been used by others to study cell death and mechanoprotection by filamin A in connective tissues after challenge by applied tensile forces (28). Since periodontal ligament fibroblasts (PDLFs) are considered to exhibit more specialized functions for repair and regeneration of the adjacent alveolar bone and cementum (29, 30), as well as adaptive roles in tooth movement in response to mechanical forces (24, 31), we postu-
lated that these cells are likely to exhibit a reduced level of apoptosis compared with gingival fibroblasts (GFs). Indeed our recent study has demonstrated a significantly lower apoptotic rate in PDLFs compared with GFs (27). However, the potential role of SFRP1 in the regulation of apoptosis for fibroblast cells remains to be established. Given this significant differential expression of SFRP1 in PDLFs compared with GFs, the aim of the present study was to comprehensively address the role of SFRP1 in the regulation of apoptosis in fibroblastic cell populations and determine the significance of its effect on apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and reagents were purchased from Invitrogen. C2-ceramide (N-acetylglucosamine) and C4-diiodoerhodamine (N-erythro-N-acetylglucosamine) were from BIoMOL Research Laboratory (Plymouth Meeting, PA). Recombinant human SFRP1 was a generous gift from Dr. Jeffrey Rubin from the NCI, National Institutes of Health. Rabbit anti-human/mouse SFRP1 antibody, rabbit anti-mouse caspase-9 antibody, rabbit anti-mouse BAX antibody, goat anti-mouse p53 antibody, goat anti-human/mouse actin antibody, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated donkey anti-goat IgG, and Western blotting laminig reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Diaminobenzidine substrate was purchased from Dako. All other chemicals were purchased from Sigma or Fisher Scientific.

**Cell Culture**—Human PDLFs and GFs were obtained, cultured, and validated as described previously (32). All the procedures were performed with appropriate informed consents and were approved by the Institutional Review Board at Goldman School of Dental Medicine. Rabbit anti-human/mouse SFRP1 antibody, rabbit anti-mouse caspase-9 antibody, rabbit anti-mouse BAX antibody, goat anti-mouse p53 antibody, goat anti-human/mouse actin antibody, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated donkey anti-goat IgG, and Western blotting laminig reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Diaminobenzidine substrate was purchased from Dako. All other chemicals were purchased from Sigma or Fisher Scientific.

**Reverse Transcription (RT)-PCR and RNase Protection Assay (RPA)**—Total RNA was isolated from cultured human PDLFs and GFs with RNalater isolation columns (Qiagen, Valencia, CA) according to the standard protocol. For the RT-PCR, 0.2 μg of total RNA was used as template for reverse transcription, and PCR was performed for 25 cycles within the linear range of amplification. For the RPA, probe synthesis and RNA hybridization were carried out using the RibonQuant RPA system (Pharmingen). Briefly, RNA probes were made by in vitro transcription in the presence of [α-32P]UTP. Labeled riboprobes were hybridized to target RNAs (20 μg/assay in all experiments) at 56 °C overnight followed by RNase A/T1 digestion at 30 °C for 45 min. Protected fragments were resolved on 6% denaturing polyacrylamide gels. Radioactive signals were recorded and quantified with a Phospho-Imager using ImageQuant software (Amersham Biosciences).

**Immunoblotting and Immunohistochemistry**—Protein contents in cultured human PDLFs and GFs were obtained as described previously (32). For secreted proteins, spent medium was concentrated, and the plates were incubated for 4 h under the culture conditions. Colored MTS products in the supernatant were then determined on a microplate reader (Bio-Rad) at 590 nm. To assess apoptotic cell death, DNA fragmentation was determined by cellular DNA fragmentation enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer’s instructions. Both attached cells and detached cells floating in the medium were collected by scraping and centrifuging. The BrdUrd-labeled DNA fragments in cytosolic fractions were quantified using a microplate reader (Bio-Rad) at 450 nm.

**Caspase-3 Measurement**—After different treatments, caspase-3 in cytosolic fractions was quantified by an ApoAlert caspase-3 colorimetric system (Clontech) according to the manufacturer’s instructions. The colorimetric assay uses the spectrophotometric detection of the chromophore p-nitroanilin (pNA) after its cleavage by caspase-3 from the labeled caspase-specific substrate (DEVD-pNA). Briefly, cells or tissues were suspended in chilled lysis buffer provided in the assay kits. After centrifugation, the supernatants were transferred to 96-well microtiter plates, and DEVD-pNA substrate was added for the detection of caspase-3 activity at 405 nm. For each assay, a standard curve with known pNA concentration was generated, and the measured absorbance based on replicates demonstrated an average of less than 5% variance.

**Apoptotic Gene Expression by cDNA Array**—Cells were cultured as described above, and total RNA was isolated after different treatments. Two test groups were included: 1) cells undergoing Cer-induced apoptosis in the presence of exogenous SFRP1 (10 μg/ml) and 2) cells undergoing Cer-induced apoptosis pretreated with siRNA (10 μM) to knock down endogenous SFRP1 expression. Cells treated only with Cer (20 μM) were used as control. Micrograms of total RNA were used for each reverse transcription step, and labeled cDNA was hybridized at 68 °C for 16 h with nylon membranes for human apoptosis gene array (Super Array, Frederick, MD). The signals were quantified by a Phosphor-Imager (Amersham Biosciences) and were normalized to glyceraldehyde-hyde-3-phosphate dehydrogenase gene, and the ratios of test and control were expressed as fold increase or decrease. A minimum of 2-fold change was considered statistically significant.

**Evaluation of SFRP1 Expression in Force-induced Cell Death**—An experimental model of tooth movement was used to study the biological relevance of SFRP1 expression during force-induced apoptosis in periodontal ligament. Eight-week-old C57B/L6J mice (20–25 g) were divided into four groups, each containing five animals. The first group served as base-line controls, which did not receive any treatment and were used as day 0 specimens. The other three groups were experimental groups in which an elastic rubber band (0.5-inch diameter) was placed between the left first and second molars of the maxilla. Three time intervals (one per group) were used for tissue collection: 12 h, 1 day, and 2 days. All animal procedures and manipulations were ap-
proved by the Institutional Animal Care and Use Committee at Boston University.

For the collection of tissues after treatments, mice were sacrificed with an overdose of ether anesthesia, and their tissues were fixed by intracardiac perfusion with a mixture of 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The dissected maxillae were decalcified in 10% EDTA solution. Maxilla tissues were cut in half along the palatal suture and then embedded using HistoPrep (Fisher Scientific). Serial mesiodistal sections (7 μm) were used for histochemical analysis, and the processing of the tissue was as described previously (35). The other half of the tissue was placed in 10% formaldehyde solution and embedded in paraffin. Sections (7 μm) parallel to the long axis of the teeth were made by cryostat sectioning. Histo logical examination was focused on the inter-radicular septum of the second molars.

**In Situ Detection of Apoptosis**—The assessment of apoptosis in vivo was performed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays (Roche Diagnostics) according to the manufacturer's instructions. For each section, the areas containing the cells were quantified using Image-Pro Plus Version 4.0 (Media Cybernetics, Silver Spring, MD). Only spindle-shaped cells were counted at 400× magnification and the number of TUNEL-positive cells/mm² was obtained.

**Effect of SFRP1 during Dermal Fibroblast Apoptosis in Vivo**—Eight-week-old C57BL/6J mice were used for a calvarial injection model to test the effect of SFRP1 on dermal fibroblast apoptosis in vivo. Briefly, the injections were administered subcutaneously with a 30.5-gauge needle at a point on the midline of the skull located between the ears and eyes. The injection volume and concentration of the Cer was determined by preliminary experiments. The control group only received a 50-μl Cer (100 μM) injection. The experimental groups were injected first with 25 μl of Cer (200 μM); this was immediately followed by a same-site injection (25 μl/mice) of 5 μg/ml BSA or recombinant SFRP1 protein at a concentration of 5 or 20 μg/ml. Twenty-four hours after each treatment, mice were sacrificed, and the calvarial bone together with the overlying skin tissue was dissected in half. One-half of the tissue was used for histological analysis, and the processing of tissues was as described previously (35). The other half of the tissue was immediately flash frozen in liquid nitrogen and stored at −80 °C for subsequent protein assays.

**Statistical Analysis**—For quantitative studies, means and S.D. were computed. Two sample comparisons were analyzed by Student’s unpaired t test, and statistical significance was set at p < 0.05.

**RESULTS**

**Level of SFRP1 Expression in PDLFs**—We analyzed the expression of SFRP1 in cultured human PDLFs and GFs at both the RNA and protein level. RT-PCR revealed significantly higher levels of SFRP1 transcripts (−18-fold) in PDLFs than in GFs at two different passages (Fig. 1A). The levels of SFRP1 transcripts were also determined by RPA, confirming a similarly high level of SFRP1 expression (−13.1-fold) in PDLFs compared with GFs (Fig. 1B). These results are consistent with our previous data on the investigation of gene expression patterns between cultured human PDLFs and GFs (32). As measured by Western blot, the cytosolic protein level of SFRP1 was also much higher in PDLFs than in GFs (Fig. 1C). However, the secreted SFRP1 in the medium was not detectable in either PDLFs or GFs (data not shown). These results suggest that SFRP1 accumulates intracellularly in PDLFs but is not secreted under normal conditions.

**Expression of SFRP1 during Cer-induced Apoptosis**—Studies have shown that sphingolipids such as ceramide are important mediators of apoptosis and growth arrest (36, 37). Our data indicated that both PDLFs and GFs undergo apoptosis after Cer exposure in a time- and dose-dependent manner (see supplemental data). Based on that, we elected to use Cer-induced apoptosis as a model to study the role of SFRP1. We have measured the mRNA levels of SFRP1 in cultured PDLFs...
and GFs after Cer-induced apoptosis at different time points by RT-PCR (Fig. 2A). A significant increase of SFRP1 mRNA was observed in PDLFs after apoptotic induction. It peaked at 24 h and remained at a high level 48 h postapoptotic induction. However, the mRNA level of SFRP1 in GFs was undetectable in unchallenged cells. It showed a small increase at 6 h followed by a gradual decrease, reaching an undetectable level again 48 h postapoptotic induction. Protein levels of cytosolic and secreted SFRP1 in cultured PDLFs and GFs were also determined by Western blotting. The results showed a significant increase of both cytosolic and secreted SFRP1 in PDLFs after Cer-induced apoptosis. This increase was first detected in the cytosolic compartment and observed later in the medium, and both cytosolic and secreted SFRP1 remained elevated 48 h postapoptotic induction. This demonstrates that SFRP1 first accumulates intracellularly and then is secreted extracellularly after Cer-induced apoptosis. However, GFs showed a minimal level of cytosolic SFRP1 in unchallenged cells and a small and short-lived increase of cytosolic SFRP1 after Cer treatment. The secreted SFRP1 protein was barely detectable in GFs throughout the indicated times.

Expression of SFRP1 during Force-induced Apoptosis—Studies have shown that periodontal ligament cells undergo apoptosis in the early phase of orthodontic tooth movement (38, 39). To assess the biological relevance between SFRP1 expression and the onset of apoptosis, we generated an experimental model using force-induced tooth movement. An elastic rubber band (3/16-inch diameter) was placed between the left first and second molars of the maxilla, and the maxilla tissues were collected at different time points: 12 h, 1 day, and 2 days. Serial mesiodistal sections (7 μm) parallel to the long axis of the teeth were made by cryostat sectioning. The assessment of apoptosis was performed using TUNEL assay. The sections were then treated with HRP-conjugated goat anti-rabbit IgG, and the slides were developed with diaminobenzidine substrate followed by counterstaining with hematoxylin. Arrows show SFRP1-positive staining. For each section, the areas containing the cells were quantified using Image-Pro Plus Version 4.0 (Media Cybernetics). Only spindle-shaped cells were counted at 400× magnification and the number of positive cells/mm² was obtained. B, root; P, periodontal ligament; B, alveolar bone.

Role of SFRP1 on Cell Viability and DNA Fragmentation—To test the effect of exogenous SFRP1 on the regulation of apoptosis, we measured cell viability and the level of DNA fragmentation in cultured human PDLFs and GFs after Cer-induced apoptosis in the presence or absence of recombinant SFRP1 (10 μg/ml). As shown in Fig. 4A, the percentage of cell survival remained the same in PDLFs in the presence or absence of exogenous SFRP1 at all time points. However, the percentage of cell survival increased greatly in GFs 12 h after...
Colored MTS products in the supernatant were determined at 490 nm.

The level of DNA fragmentation was determined by enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer’s instructions. The resulting double-stranded siRNA (10 nM) was transfected into the cultured cells using siPORT amine transfection reagent (Ambion). A, cellular total RNA was extracted at different time points, and the level of SFRP1 transcript was detected by RT-PCR. Human β-actin gene was used as an internal control. B, cell lysate was extracted at different time points, and the protein level of SFRP1 was detected by Western blot. Equal loading of cell lysates was confirmed by probing the blot with an anti-actin antibody. sFRP1L, SFRP1 from cell lysates.

**Fig. 5.** Depletion of endogenous SFRP1 by RNA interference. The expression and purification of siRNA for SFRP1 were performed using the Silencer siRNA construction kit (Ambion) following the manufacturer’s instruction. The resulting double-stranded siRNA (10 nM) was transfected into the cultured cells using siPORT amine transfection reagent (Ambion). A, cellular total RNA was extracted at different time points, and the level of SFRP1 transcript was detected by RT-PCR. Human β-actin gene was used as an internal control. B, cell lysate was extracted at different time points, and the protein level of SFRP1 was detected by Western blot. Equal loading of cell lysates was confirmed by probing the blot with an anti-actin antibody. sFRP1L, SFRP1 from cell lysates.

**Fig. 4.** Effect of exogenous SFRP1 on cell viability and DNA fragmentation. Cells were grown in 6-well culture plates until they reached 60–70% confluence in DMEM, 10% fetal bovine serum. The cells were then washed with phosphate-buffered saline and changed to low serum (1%) DMEM. One hour after the addition of Cer (20 μM), human recombinant SFRP1 protein was added to the cell cultures at a concentration of 10 μg/ml for the times indicated. As a control, 10 μg/ml BSA was added to the cultures instead of SFRP1 protein. A, cell viability was measured using the CellTiter 96 proliferation assay (Promega). Colored MTS products in the supernatant were determined at 490 nm. B, the level of DNA fragmentation was determined by enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer’s instructions. The BrdUrd-labeled DNA fragments in cell lysates were quantified at 450 nm. Data are presented as mean ± S.D. of three independent repeats and expressed as absorbance ratio relative to the control groups (BSA-treated). *p < 0.05, difference between PDLFs and GFs.

The addition of SFRP1 as compared with control BSA (81.5 versus 52.8% at 12 h, 75 versus 38.1% at 24 h, and 58.5 versus 27.2% at 48 h). The effect of exogenous SFRP1 was also evaluated by the detection of DNA fragmentation in cultured human PDLFs and GFs (Fig. 4B). The level of DNA fragmentation was significantly decreased in GFs after the addition of SFRP1 as compared with control (A450 nm: 0.817 versus 1.27 at 24 h and 0.969 versus 1.49 at 48 h), whereas no changes were observed in PDLFs under the same treatments. This suggests that the constitutive up-regulation of SFRP1 in PDLFs contributes maximally to the inhibition of apoptosis in PDLFs such that additional SFRP1 has minimal effect. In contrast, exogenous SFRP1 may have substantial antiapoptotic effects on GFs, which do not maintain high levels of endogenous SFRP1 expression.

We also synthesized 21-mer double-stranded siRNA molecules complementary to the SFRP1 coding sequence and transfected them into the cultured PDLFs and GFs. siRNA used in the experiment significantly inhibited endogenous SFRP1 expression in PDLFs at both mRNA and protein levels (Fig. 5, A and B). This provided us with a helpful tool to study the role of endogenous SFRP1 on the regulation of apoptosis. After pre-treatment with siRNA for SFRP1 inhibition, the percentage of cell survival (Fig. 6A) and the level of DNA fragmentation (Fig. 6B) were investigated in cultured PDLFs and GFs after Cer-induced apoptosis. The percentage of cell survival was decreased significantly in PDLFs 24 h after the knock-down of SFRP1 expression (43 versus 62.9% at 24 h and 28.1 versus 41.7% at 48 h), and the DNA fragmentation level was greatly elevated 12 h after the inhibition of SFRP1 expression (A450 nm: 1.06 versus 0.64 at 12 h, 1.86 versus 1.06 at 24 h, and 2.47...
versus 1.31 at 48 h). As expected, no changes were observed in GFs in cell viability and level of DNA fragmentation in the presence or absence of siRNA, probably due to the minimal level of endogenous SFRP1 expression in GFs.

**Apoptotic Gene Expression by cDNA Array**—To gain insights into the effects of gain or loss of SFRP1 function in the regulation of apoptosis, we investigated the expression pattern of apoptosis-related genes in cultured human PDLFs and GFs after Cer-induced apoptosis using a pathway-specific cDNA microarray. A number of apoptosis-related genes were differentially expressed (≥2-fold) in PDLFs when SFRP1 function was enhanced by addition of SFRP1 protein or inhibited by RNA interference. There were also genes that were differentially expressed in GFs when SFRP1 function was enhanced by addition of SFRP1 protein (see supplemental data). As shown in Table I, we observed that the mRNA expression of a set of apoptosis-related genes was regulated to the opposite direction when SFRP1 function was enhanced or inhibited, and such regulation was consistent in both PDLFs and GFs. For instance, while the addition of SFRP1 protein down-regulated caspase-3 gene expression in PDLFs by 2.17-fold, the inhibition of SFRP1 expression up-regulated the caspase-3 gene expression in PDLFs by 7.27-fold. Furthermore, the down-regulation of caspase-3 gene was also observed in GFs (3.13-fold) after the addition of SFRP1 protein. These data strongly suggest that the antiapoptotic effect of SFRP1 could involve the regulation of these common apoptosis-related genes: p53, caspase-3, caspase-9, and BIK.

**Role of SFRP1 on Caspase-3 Activity**—Since caspase-3 is the primary activator of DNA fragmentation, we studied the effect of SFRP1 on the caspase-3 enzyme activity after Cer-induced apoptosis. The addition of SFRP1 slightly decreased active caspase-3 level in cultured human PDLFs at the 12 and 24 h time points compared with the control group; however, this was not statistically significant (Fig. 7A). In contrast, a significant decrease of caspase-3 activity was observed in GFs 12 h after the addition of SFRP1 as compared with the control group (Fig. 7B). On the other hand, the SFRP1 gene knock-down by RNA interference caused a significant elevation of caspase-3 activity in PDLFs as early as 6 h post-siRNA transfection (Fig. 7C). However, there was little difference observed in GFs before and after RNA interference for SFRP1 (Fig. 7D). These data together with the array results suggest that the effect of SFRP1 on the enhancement of cell survival might be achieved through the inhibition of caspase-3 activity.

**Antiapoptotic Effect of SFRP1 in Dermal Fibroblasts**—To extend our understanding of the role of SFRP1 on the regulation of fibroblast apoptosis, we tested the antiapoptotic effect of SFRP1 on a general fibroblast population, i.e. dermal fibroblasts. As shown in Fig. 8, addition of BSA had no effect on Cer-induced apoptosis (p > 0.05) as the number of TUNEL-positive cells remained the same compared with the control group (114.7 ± 22.2 versus 128.4 ± 32.5 cells/mm²). However, the number of TUNEL-positive cells decreased to 86.2 ± 20.5 cells/mm² when 5 μg/ml SFRP1 protein were injected. Injection of 20 μg/ml SFRP1 showed a significant inhibition of Cer-induced apoptosis (p < 0.01) in dermal fibroblasts (15.7 ± 5.1 cells/mm²) when compared with the control group. To further determine the potential targets of SFRP1 in these tissues, the expression levels of genes shown in Table I were measured by Western blot (Fig. 9, A and B). Due to the unavailability of the anti-mouse BIK antibody, we elected to detect the expression of BAX, a gene that was shown to be down-regulated by the addition of SFRP1 in the array results (see supplemental data). The p53 protein in the tissue was up-regulated by 4- and 7-fold, respectively, after the injection of 5 and 20 μg/ml SFRP1 as compared with the control group. Caspase-9 protein levels were decreased by 2.6-fold in the presence of 5 μg/ml SFRP1, while a higher concentration of SFRP1 (20 μg/ml) did not further reduce this level of expression. Meanwhile BAX protein level was reduced by 2-fold after the injection of 5 μg/ml SFRP1, while a 4-fold reduction of BAX expression was observed after 20 μg/ml SFRP1 was added. When compared with the control, the injection of BSA did not change the expression level of tested proteins except for a slight reduction of BAX expression. Furthermore the effect of SFRP1 on the caspase-3 enzyme activity was also checked in the extracted tissues (Fig. 9C). The addition of BSA or a low concentration of SFRP1 (5 μg/ml) had little effect on caspase-3 activity (p > 0.05). In contrast, significant decrease of caspase-3 activity was detected after the injection of 20 μg/ml SFRP1 when compared with the control group.

**DISCUSSION**

Dermal fibroblasts are the predominant cells of the periodontal ligament and the gingiva, and they have important roles in the function and tissue regeneration of the tooth-supporting apparatus. Although PDLFs and GFs resemble each other morphologically, a substantial functional heterogeneity exists between them. Our previous study (27) suggested that PDLFs are likely to have a lower rate of turnover compared with GFs to achieve more prolonged and specialized functions for repair and regeneration of the adjacent alveolar bone and cementum (29, 30) as well as adaptive roles in tooth movement in response to applied forces (24, 31).

Currently little is known about the potential role of SFRP1 in apoptosis of fibroblast-related cells (e.g. dermal, periodontal, or gingival fibroblasts). We tested the role of SFRP1 in the regulation of apoptosis in PDLFs versus GFs both in vitro and in vivo. Our data showed that SFRP1 was expressed at a significantly higher level in cultured human PDLFs compared with GFs during the induction of apoptosis. In vitro study using a tooth movement model further verified the biological relevance of SFRP1 expression during force-induced apoptosis in periodontal ligament. Inhibition of endogenous SFRP1 expression significantly increased the level of Cer-induced apoptosis in PDLFs, whereas exogenous SFRP1 substantially inhibited apoptosis in GFs, which do not maintain a high level of endogenous SFRP1 expression. Together these observations suggest that SFRP1 expression and function are essential for the control of

| Symbol | GenBank™ accession number | Description | PDLFs/+/− Fold Up/down | PDLFs/−/− Fold Up/down | GFs/+ Fold Up/down |
|--------|---------------------------|-------------|------------------------|------------------------|-------------------|
| TP53   | M14694                    | Tumor protein p53 | 2.9 ▲                   | 5.2 ▼                   | 5.5 ▼ |
| CASP3  | NM_004346                 | Caspase-3    | 2.17 ▲                  | 7.27 ▼                  | 3.13 ▼ |
| CASP9  | U05521                    | Caspase-9    | 2.2 ▼                   | 2.36 ▲                  | 4.71 ▼ |
| BIK    | U34584                    | BCL-2-interacting killer | 4.35 ▼                  | 5.43 ▲                  | 2.04 ▼ |

\( ^\mathrm{a} + \), addition of SFRP1 protein.  
\( ^\mathrm{b} _{-} \), inhibition of SFRP1 expression by RNA interference  
\( ^\mathrm{c} \), Up-regulation or down-regulation of gene expression relative to control.
cell turnover in PDLFs, and there may be a differential effect of the endogenous versus exogenous SFRP1 on the prevention of fibroblast cell apoptosis. Since SFRP1 has been suggested to interfere with Wnt signaling molecules, which can regulate gene expression in both autocrine and paracrine fashions (40, 41), it was reasonable to believe that both autocrine and paracrine effects could be involved in the SFRP1-induced inhibition of cell apoptosis.

Our data demonstrated that the contribution of SFRP1 to the enhanced cell survival in PDLFs may be achieved through the down-regulation of the proapoptotic BCL-2 genes (BIK and BAX) and the subsequent inhibition of caspase activity (caspase-9 and caspase-3). BIK is a tissue-specific, BCL-2 homology domain 3 (BH3)-only protein that promotes cell death in a manner similar to the death-promoting members of the BCL-2 family, BAX and BAK (5). Although BIK does not interact with BAX, BIK-induced cell death is mediated by an entirely BAX-dependent mitochondrial pathway (42). In BAX-positive cells, BIK interacts with the cellular survival-promoting proteins, BCL-2 and BCL-xL, and induces a conformational switch in the BAX leading to cytochrome c release, mitochondrial permeability transition, and caspase activation (42). Indeed our results substantiated the correlation of BIK with BAX, showing a down-regulation of BAX gene in the presence of SFRP1. Our results also showed a differential expression of caspase-9 and inhibition of caspase-3 activity upon addition of SFRP1. This suggests that SFRP1 may reduce fibroblast apoptosis through the inhibition of BAX-induced caspase activation and apoptosis (43).

Interestingly an up-regulation of p53 gene was observed after the addition of SFRP1 during apoptotic induction. Depending on cell type or context, wild-type p53 limits cellular proliferation by inducing cell cycle arrest, apoptosis, or senes-
cence (44, 45). To date, a dual function of p53 on apoptosis has been suggested. p53 protein can induce apoptosis via the induction of several target genes involved in the mitochondrial apoptotic pathway. Several mediators of the p53-induced apoptotic response are proapoptotic members of the BCL-2 family, such as BIK and BAX (46, 47). On the other hand, recent reports have demonstrated a novel type of gene targeted by p53, such as heparin-binding epidermal growth factor-like growth factor, which is induced as a cellular stress response and lessens p53-mediated apoptosis through Ras/Raf/mitogen-activated protein kinase activation (48, 49). The observed down-regulation of BIK and BAX by SFRP1 suggests that the SFRP1-induced up-regulation of p53 does not lead to the ultimate activation of mitochondrial apoptotic pathway. A possible explanation is the intervention by BCL-2 gene, which was observed to be up-regulated in the presence of SFRP1. Studies have shown that BCL-2 is involved in the determination of p53-dependent survival versus apoptosis and can promote p53-dependent senescence without affecting the G1/S transition (50, 51). Thus up-regulation of BCL-2 by SFRP1 could inhibit the p53-dependent mitochondrial apoptotic pathway. Furthermore, since the overexpression of BCL-2 turns the apoptotic phenotype into a senescent-like phenotype that is resistant to apoptosis, it was reasonable to believe that SFRP1-induced up-regulation of p53 could be an important mechanism for cell survival during senescence. However, the effect of SFRP1-induced up-regulation of p53 on Ras/Raf/mitogen-activated protein kinase activation still remains to be determined.

In addition, data obtained from the calvarial injection model revealed the antiapoptotic effect of SFRP1 on dermal fibroblasts during apoptotic induction. This finding extends our understanding of the role of SFRP1 on the regulation of apoptosis in general fibroblast population. If SFRP1 is identified as an intrinsic cell survival marker for fibroblastic cells, understanding of its underlying mechanism could allow the development of therapeutic strategies for soft connective tissue repair and regeneration.

Overall, this study sheds new light on the mechanistic control of fibroblast apoptosis, suggesting that SFRP1 contributes to the reduced level of fibroblast apoptosis through the regula-

Fig. 9. Analyses of mice calvarial tissues. A, after different treatment, protein extractions from mice calvarial tissues were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Each of the primary antibodies against p53, caspase-9, BAX, and actin was added in Tris-buffered saline/Tween-20 containing 5% BSA and was incubated with the membrane overnight at 4 °C. Membranes were then incubated with HRP-conjugated secondary antibody and developed by the ECL system. B, Western blots for each tissue sample were analyzed by densitometry, and the expression of each protein was expressed as a ratio relative to actin. C, after different treatment, solubilized calvarial tissues were suspended in chilled cell lysis buffer provided in the ApoAlert caspase-3 colorimetric assay kits (Clontech). After centrifugation, the supernatants were transferred to 96-well microtiter plates, and DEVD-pNA substrate was added for the detection of caspase-3 activity at 405 nm. Data are presented as mean ± S.D. of three repeats. *, p < 0.05, relative to Cer-only group.
tion of p53, caspases, and BAX. Local administration of SFRP1, including application of recombinant SFRP1 or inhibition of endogenous SFRP1 expression, could be a useful therapeutic approach to enhance wound healing and tissue regeneration by specifically modifying fibroblastic cell turnover.

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