Proteomic Analysis of Gingival Tissue and Alveolar Bone during Alveolar Bone Healing*§

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Bone tissue regeneration is orchestrated by the surrounding supporting tissues and involves the build-up of osteogenic cells, which orchestrate remodeling/healing through the expression of numerous mediators and signaling molecules. Periodontal regeneration models have proven useful for studying the interaction and communication between alveolar bone and supporting soft tissue. We applied a quantitative proteomic approach to analyze and compare proteins with altered expression in gingival soft tissue and alveolar bone following tooth extraction. For target identification and validation, hard and soft tissue were extracted from mini-pigs at the indicated times after tooth extraction. From triplicate experiments, 56 proteins in soft tissue and 27 proteins in alveolar bone were found to be differentially expressed before and after tooth extraction. The expression of 21 of those proteins was altered in both soft tissue and bone. Comparison of the activated networks in soft tissue and alveolar bone highlighted their distinct responsibilities in bone and tissue healing. Moreover, we found that there is crosstalk between identified proteins in soft tissue and alveolar bone with respect to cellular assembly, organization, and communication. Among these proteins, we examined in detail the expression patterns and associated networks of ATP5B and fibronectin 1. ATP5B is involved in nucleic acid metabolism, small molecule biochemistry, and neurological disease, and fibronectin 1 is involved in cellular assembly, organization, and maintenance. Collectively, our findings indicate that bone regeneration is accompanied by a profound interaction among networks regulating cellular resources, and they provide novel insight into the molecular mechanisms involved in the healing of periodontal tissue after tooth extraction. *Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.026740, 2674–2688, 2013.

Healthy dental gingival tissue and the alveolar bone that surrounds the teeth are essential for the proper function of teeth, as well as for a good appearance and good general health. Socket healing after tooth extraction is a useful experimental model for investigating the communication between gingival tissue and alveolar bone after tooth extraction. Preservation of the alveolar socket after tooth extraction requires the formation of a biological connection between the living and osseous tissue, which has to be created during the healing process. The success of such dental remodeling is dependent on the establishment of a soft tissue barrier that is able to shelter the underlying osseous structures and the osseo-integration of the soft tissue surrounding the alveolar bone. Understanding the processes governing soft and hard tissue healing and maintenance around the alveolar socket is paramount for oral health.

Several studies have reported significant structural changes and bone reabsorption in fresh sockets following tooth extraction, with important dimensional changes in the surrounding alveolar bone (1–3). A reduction of alveolar bone may present problems after tooth extraction, especially in aged individuals in whom bone volume is important for both physiological and medical reasons. Although it has been shown that reduction defects in alveolar bone can be completely repaired using surgical techniques such as guided bone regeneration (4, 5), bone autograft, bone allograft, and xenograft (6, 7), these techniques are not broadly applicable (8). However, the introduction of biomimetic agents such as enamel matrix derivatives (9), platelet-rich plasma (10), platelet-derived growth factor (11, 12), and bone morphogenic proteins (BMPs)1 (9) promises potentially better outcomes with

1 The abbreviations used are: ATP5B, ATP synthase subunit beta, mitochondrial; BGN, biglycan; BMP, bone morphogenetic protein; DCN, decorin; ECM, extracellular matrix; FN1, fibronectin 1; MS, high/low-collision-energy mass spectrometry; SERPINF1, serpin peptidase inhibitor, clade F, member 1; TGF-β, transforming growth factor β; UPLC, ultraperformance liquid chromatography.
bone regeneration treatments, although their efficacy remains controversial.

The proteins present in bone are essential for all of the life processes ongoing in bone, and they are the most important final products of the homeostatic signaling pathways. Profiling those proteins is vital for a thorough understanding of bone biology. To date, proteome research on bone has been focused mainly on in vitro analysis of bone-forming cells (osteoblasts and osteoclasts) to determine which proteins are expressed under a given set of experimental conditions (13–16). Although important, such studies cannot identify the actual protein profile in oral alveolar bone. Recently, the extraction of proteins directly from skull bone for proteome analysis was reported (17, 18). The extracted proteins were first separated using two-dimensional gel electrophoresis, after which spots of interest were excised and the proteins were identified via mass spectrometry (MS). However, using two-dimensional gel electrophoresis to analyze extreme proteins (e.g., extremely basic or acidic, extremely small or large, extremely hydrophobic) is challenging. Shotgun proteomics, which is a method of high-throughput proteome analysis (19–21), avoids the intrinsic limitations of two-dimensional gel electrophoresis. Despite an interesting need for large-scale characterization of the bone proteome, one study has been reported to apply shotgun proteomics for proteome analysis of rat femur bone (22). However, they identified only 133 proteins, because they analyzed bone proteins using a one-step method without a demineralization stage. The other report showed only that bone proteins extracted from the skull bone of an adult beagle are carried using a demineralization step (23).

There are no reports regarding the interaction between alveolar bone and soft tissue yet.

The efficient extraction of bone proteins is a critical issue for proteome analysis (24). Because bone is largely mineralized, and therefore nearly solid, classical protein extraction methods used for soft tissues and cells may not be appropriate for bone. It is therefore necessary to develop methods to efficiently extract protein from bone. In earlier bone proteome analyses (17, 18, 22), the bones were first ground to powder, after which the proteins were extracted by means of incubating the powder in lysis buffer. However, mechanically breaking bones down into powder is laborious, especially for large animal bones. More important, large amounts of collagen and proteoglycans also are extracted, and this can impair the detection of low-abundance proteins and strongly affect iso-electric focusing (25). For the present study, we adopted an alternative method of demineralizing bone tissue and then investigated the efficiency of protein extraction from the demineralized bone tissue. This method was based on a recently reported sequential protein extraction protocol that was used to extract proteins from skull for comprehensive analysis of its proteome. Two-dimensional high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) was then applied to analyze the protein extracts, enabling the identification of 2479 proteins (23). We employed a similar method to extract and identify proteins in tooth alveolar bone.

Given that a large number of proteins are likely involved in the healing of bone, as well as of soft tissues, another goal of the present study was to examine protein expression and putative signaling during bone healing after tooth extraction. Here, we used nano-UPLC-MS^2-based label-free quantitative proteomics to analyze alveolar bone and the adjacent soft tissue. The environment surrounding healing bone would be expected to affect the specific signaling networks involved in bone regeneration. We suggest that determining the protein networks in alveolar bone and gingival tissue will enable improvement of the soft tissue interface, aspects of the hard tissue, and dental appearance during and after therapy.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—All chemicals were purchased from Sigma-Aldrich, Merck, or USB (Affymetrix, Inc., Santa Clara, CA).

**Animal Experimental Groups**—Mini-pigs (miniature pig; PWG Genetics Korea, Ltd) were maintained under specific-pathogen-free conditions. All animal-related procedures were reviewed and approved under the Animal Care Regulations of Chonnam National University (accession number: CNU IACUC YB-2011-3). For proteomic analysis, nine pigs were subdivided into three groups (n = 3 in each group) and were sacrificed without tooth extraction, 1 week after tooth extraction, or 2 weeks after tooth extraction, respectively. After a mini-pig was sacrificed, bone fragments were harvested from the surgical area (the first to the fourth premolars on the right and left sides of the maxillary and mandibular regions). After tooth extraction, the fragments were trimmed to separate the gingival soft tissue from the alveolar bone. Both soft and hard tissues were stored at −70 °C until analyzed.

**Preparation of Soft Tissue Proteins**—For protein extraction, gingival soft tissue was homogenized in buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 1 μg/ml leupeptin, and 1 mM PMSF. The proteins were extracted over a 4-h period at 4 °C, after which the extract was sonicated and cleared via centrifugation at 13,000 rpm for 30 min at 4 °C. The protein concentration in the cleared extract was measured using a BCA protein assay (Thermo Scientific, Rockford, IL), and samples containing 200 μg of protein were subjected to 10% SDS-PAGE. The separated proteins were then visualized by staining with Coomassie Blue R-250, and the gels were washed three times in distilled water prior to tryptic digestion. For efficient trypsinization, the separated proteins were collected from the gels by excising 10 slices of gel for each patient sample, as described previously (26). The gel slices were then chopped into 1-mm³ pieces and destained overnight via incubation in 50% acetone and 13,000 rpm for 30 min at 4 °C. The protein concentration in the cleared extract was measured using a BCA protein assay (Thermo Scientific, Rockford, IL), and samples containing 200 μg of protein were subjected to 10% SDS-PAGE. The separated proteins were then visualized by staining with Coomassie Blue R-250, and the gels were washed three times in distilled water prior to tryptic digestion. For efficient trypsinization, the separated proteins were collected from the gels by excising 10 slices of gel for each patient sample, as described previously (26). The gel slices were then chopped into 1-mm³ pieces and destained overnight via incubation in 50% acetoni

**Preparation of Alveolar Bone Proteins**—Protein was extracted from the gels slices by excising 10 slices of gel for each patient sample, as described previously (26). The gel slices were then chopped into 1-mm³ pieces and destained overnight via incubation in 50% acetonitrile and 1 mM ammonium bicarbonate. Each gel piece was then reduced using 10 μM dithiothreitol and alkylated using 55 mM N-ethylmaleimide in 100 mM ammonium bicarbonate. Following trypic digestion (2 μg/sliced gel; Promega, Madison, WI) for 16 h at 37 °C, the peptides were recovered and extracted from the sliced gels using 5% formic acid and 50% acetonitrile. After extraction, the peptides were dried in a vacuum centrifuge and combined into a single tube. The combined samples were desalted using a solid-phase Oasis HLB C18 microelution plate (Waters, Inc., Milford, MA) and then stored at −80 °C until subjected to nano-LC-MS/MS for comparative proteomics.

**Preparation of Alveolar Bone Proteins**—Protein was extracted from alveolar bone using the method described by Jiang et al., with some modifications (Fig. 1A) (23). Alveolar bone fragments (about 10 mm³
long × 10 mm wide × 10^{-20} \text{mm thick} were trimmed of soft tissue in liquid nitrogen and then incubated overnight in PBS (pH 7.4) containing a protease inhibitor mixture at 4 °C to remove contaminants. Thereafter, about 150 mg of bone fragments were incubated in 1.2 M HCl overnight at 4 °C to demineralize the bone (200 mg of bone slice per milliliter of solution). After centrifugation, the supernatant was collected as Extract 1. The pellet was washed with water and incubated in buffer containing 100 mM Tris, 6 M guanidine-HCl (pH 7.4), and a protease inhibitor mixture for 72 h at 4 °C. After centrifugation, this supernatant was collected as Extract 2. The pellet was extracted further in the extraction solution (100 mM Tris, 6 M guanidine-HCl, pH 7.4) containing 0.5 mM tetrasodium EDTA for 72 h at 4 °C, and the supernatant was collected as Extract 3 after centrifugation. Finally, the remaining pellet was incubated in 6 M HCl at 4 °C, and the supernatant was collected as Extract 4 after centrifugation. All centrifugations carried out during the extraction protocol were at 12,000 rpm for 20 min at 4 °C, and all crude protein extracts were precipitated with acetone overnight at −20 °C. The four samples of precipitated protein (Extracts 1–4) were then separately dissolved in 200 μl of buffer containing 100 mM Tris and 6 M guanidine-HCl (pH 8.1). Once in solution, the extracts were combined (total volume: 800 μl) and the protein concentration was determined using Bradford assays (Bio-Rad Laboratories), after which 200-μg aliquots of protein were subjected to 10% SDS-PAGE. Thereafter, tryptic digestion was carried out as described for soft tissue.

**Protein Identification and Quantitative Analysis Using Nano-UPLC-MS² Tandem Mass Spectrometry**—Separations were performed on a nano-UPLC C18 RP column (75 μm × 250 mm; particle size, 1.7 μm) and an enrichment Symmetry C18 RP column (180 μm × 20 mm; particle size, 5 μm) using a nano-AQUITY Ultra Performance² chromatography system (Waters Corporation, Milford, MA). The LC gradient program and operation mode were as reported previously (27). Trypsin-digested peptides (5 μl) were loaded onto the enrichment column with mobile phase A (3% acetonitrile in water with 0.1% formic acid), and a step gradient was used at a flow rate of 300 nl/min. This included a 3%–40% mobile phase B (97% acetonitrile in water with 0.1% formic acid) over 95 min and a 40%–70% mobile phase B over 20 min, followed by a sharp increase to 80% B within 10 min. Sodium formate (1 μM) dissolving of 10 mM NaOH in isopropanol and 0.2% HCOOH (1:1, v/v) was used to externally calibrate the TOF analyzer in the range of m/z 50–2000 with series of singly or doubly charged ion clusters prior to LC-MS/MS experiments (28).

The mass accuracy of the raw data was corrected against the monoisotope ion of [Glu₁]-fibrinopeptide (m/z 785.8426 Da [M + 2H]²⁺). The [Glu₁]-fibrinopeptide (200 fmol/μl, 600 nl/min flow rate) was infused every 1 min into the mass spectrometer for lock mass correction during sample analysis. During data acquisition, the collision energy in the low-energy MS mode and in the elevated-energy MS² mode were recorded to improve the mass accuracy of the parent ion. One cycle of MS and MSE was performed every 3.2 s. In each cycle, MS spectra were acquired for 1.5 s using a 0.1-s inter-scan delay (m/z 300–990), and the ions exceeding 50 counts were selected for MS² fragmentation in the collision cell (m/z 50–2000).

LC-MS² data were processed and searched using ProteinLynx GlobalServer version 2.3.3 (Waters Corporation) to reconstruct MS/MS spectra by combining all masses with identical retention time deviation threshold (<0.25 min) and mass precision (<15 ppm) in ProteinLynx GlobalServer 2.3.3. For normalization of the dataset, each sample was spiked with a tryptic digested standard protein mixture including yeast alcohol dehydrogenase (1 pmole), rabbit glycogen phosphorylase B (0.5 pmole), yeast enolase (4 pmole), and bovine serum albumin (8 pmole) (Mass PREP Digestion Standard Mixture, Part No. 186002866, Waters) as an internal standard. Yeast alcohol dehydrogenase was mainly used for normalization between datasets.

**Bioinformatics Analysis**—Ingenuity Pathway Analysis (IPA version 8.0, Ingenuity Systems Inc., Redmond City, CA) was used for knowledge-based network and canonical pathway analysis of the nano-LC-MS² comparative proteomics data with the built-in function. STRING 9.0 was queried using human protein symbols from supplemental Fig. S1. The selected parameters were set to medium confidence (0.400) and prediction options “Co-expression,” “Experiments,” and “Databases.”

**Quantitative Real-time PCR**—Total RNA was isolated from porcine gingival tissue (3 h, 1 week, or 2 weeks after tooth extraction) using QiAzo® RNA Lysis reagent (Qiagen Sciences, Valencia, CA), after which cDNAs were synthesized using a PrimeScript™ RT Reagent Kit for real-time PCR (Takara Biotechnology, Japan) according to the manufacturer’s instructions. Quantitative PCR was performed using an ABI 7300 Prism SDS real-time PCR detection system (Applied Biosystems, Foster City, CA) with a SYBR® Premix Ex Taq Tag kit (Takara Biotechnology) and a standard temperature protocol. The results obtained using a cycle threshold are expressed as relative quantities and were calculated using the 2^{-ΔΔCT} method (expressed as the relative fold ratio). Hypoxanthine phosphoribosyltransferase 1 was used as a control gene for normalization, and three separate experiments were performed. Supplemental Table S1 lists the porcine primers (Sus scrofa) used for the quantitative real-time PCR.

**Western Blotting and Immunohistochemistry**—Samples of protein extracted from porcine gingiva (soft tissues) were separated on 12% polyacrylamide gels. The separated proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories), after which the membranes were incubated first with anti-ATPB (NBP1–54700, Novus Biologicals, Littleton, CO) or anti-β-actin (A5441, Sigma-Aldrich) as the primary antibody and then with HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA). Immunoreactive proteins were detected using an ECL system (NiFRON, South Korea).

Maxillae, including the teeth, were removed and immersed for 24 h in 10% neutralized buffered formalin solution containing 0.1 mol/l phosphate buffer (pH 7.4). After decalcification in 10% EDTA solution for 4 weeks at 4 °C, the specimens were embedded in paraffin, and sagittal sections of the teeth were cut at a thickness of 4 μm. The sections were then processed for H&E staining and immunohistochemistry. All unstained slides were stained with anti-BMP2 (1:50, ab28251, Abcam), anti-osteonectin (1:50, ab8448, Abcam, Cambridge, UK), anti-ATPSB (1:50, NBP1–54700, Novus Biologicals), and anti-fibronectin (1:200, ab23751, Abcam) antibodies using a Ventana Ultraview DAB detection kit in a Ventana BenchMark XT processor (Ventana, Tucson, AZ). Antigen retrieval was not per-
formed for any antibodies. Applied antibodies were incubated for 60 min at 37 °C. As negative controls, serial sections of the same specimens were processed, substituting commercially available mouse non-immune immunoglobulin G serum (DAKO, Carpinteria, CA) for the primary antibody.

RESULTS

Experimental Strategy for Comparative Proteomics in Gingival Tissues and Alveolar Bone After Tooth Extraction—We used a porcine model with standard radiography and immunohistochemical analysis of osteogenic markers to characterize the quantitative proteomic differences in soft tissue and bone before and after tooth extraction. We found that sockets filled with particles of regenerated bone within 1 week after tooth extraction (Fig. 1B). By 2 weeks post-extraction, BMP2 immunoreactivity was present in the soft tissue, mainly in fibroblasts and adipose tissue, and osteopontin was seen in the matrix (Fig. 1C). Notably, bone regeneration actively arose after tooth extraction in this pig model, confirming the model’s
suitability for use in identifying the proteins involved in active bone healing.

The strategy we used is illustrated in Fig. 1A. In three replicates, proteins extracted from gingival soft tissue and alveolar bone were digested with trypsin, after which the digested peptides were desalted using C18 and analyzed using a label-free quantitative proteomics approach. This entailed separation of the digested materials using nano-UPLC followed by analysis online using a Q-TOF tandem mass spectrometer. For efficient separation, we used a 25-cm reversed-phase column packed with small particles (1.7 μm) combined with ultrahigh pressure (~7000 psi), which provided adequate separation in a single chromatography run. We also used MS6 technology, which entailed continuous switching between low and high collision energies to gather precursor ion masses (low collision) and fragment ions (high collision) at 1-s intervals and yielded more than five times more information from a single LC run. Using this approach to measure the peak intensities of individual peptide masses obtained in separate chromatography runs without the incorporation of stable isotope tags confirmed the reproducibility of the results obtained using nano-UPLC.

To further ensure the reliability of the quantitative profiling results, three samples (no extraction, 1 week after tooth extraction, and 2 weeks after tooth extraction) were prepared and analyzed in triplicate, which allowed the rates of expression change above 1.1-fold change and below 0.9-fold change to be determined with high significance (p < 0.01) (supplemental Fig. S2). A total of 16,961 MS/MS spectra in soft tissue and 17,163 in bone were designated as peptides and were detected more than once, leading to the confident identification of a total of 946 proteins in soft tissue and 940 in bone with an average of at least two unique peptides per protein (supplemental Table S2). From among the total identified protein sets, 340 proteins for which we had precise quantitative values in more than one of the three samples were selected from soft tissue and bone (supplemental Table S3). Table I lists 62 proteins found to be differentially expressed with fold changes ranging between more than 1.5 (ratio ≥ 1.5) and less than 0.6 (ratio ≤ 0.6) through the comparison of sample pairs (supplemental Table S4). Of these, 56 proteins were differentially expressed in gingival soft tissue and 27 proteins were differentially expressed in alveolar bone, comparing among the three samples (Fig. 2A). These proteins belong to extracellular matrix (15), plasma membrane (3), mitochondria (4), endoplasmic reticulum (3), and cytoplasm and nucleus (14). In summary, 62 identified proteins may be targets affected by bone regeneration, and vice versa.

Classification of Expression Differences between Soft Tissue and Bone—In subsequent bioinformatics analyses, we focused on the 62 proteins differentially expressed in soft tissue and bone. In order to provide an overview of the functional roles of the differentially expressed proteins, the 62 proteins were annotated based on Ingenuity functional analysis assignments (Figs. 2B and 2C). Their most prominent and relevant actions in soft tissue included MAPK/ERK-related signaling (ERK5, 14-3-3-mediated, PI3K/AKT, IGF-1, and p70S6K signaling), p38-related signaling (Myc-mediated apoptosis, 14-3-3-mediated signaling, and G2/M checkpoint regulation), and glucose metabolic processes (rectangles outlined by solid lines in Fig. 2B), whereas receptor-mediated signaling (glucocorticoid receptor and clathrin-mediated endocytosis signaling) was clearly overrepresented in bone (rectangles outlined by solid lines in Fig. 2C). Networks of the altered proteins in both soft tissue and bone after tooth extraction were related to cell–cell communication (junction signaling and integrin-linked kinase signaling), cellular maintenance (liver X receptor/retinoid X receptor activation and actin cytoskeleton signaling), and injury-inducible responses (acute phase response signaling, hepatic fibrosis activation, and coagulation system). Protein interactions between and within the affected correlations are presented as an interaction network, which also reveals that various proteins involved in gene regulation are differentially expressed in soft tissue and bone after tooth extraction. These evident effects on several fundamental and essential cellular processes demonstrate that there are profound differences between the functional networks active in soft tissue and in bone responding to bone healing. To obtain further insight into key proteomic differences, we arranged functional profiles of the proteins involved in these processes, as detailed below.

Distinct Protein Networks of Targets in Soft Tissue and Bone—To further validate the correlation among proteins showing expresisonal alteration, we evaluated the connection between associated networks of targets in soft tissue and bone (supplemental Table S5). The majority of the proteins involved in these networks were found to have disease- and disorder-related functions in soft tissue (21 proteins) and cellular organization and maintenance-related functions in bone (17 proteins). Of the 21 quantified proteins related to a disease condition, 12 (ACTN1, ACTN4, ANXA2, ATP5A1, ATP5B, EEF1A1, ENO1, LTF, PFN1, PKM, TKT, and VCP) showed a fold increase 1 week post-extraction, and 7 (FGG, FSCN1, GAPDH, PGAM1, PPIA, YWHAH, and YWHAZ) showed significantly higher levels 1 week post-extraction, relative to no extraction. At the transcriptional level, several candidates (LTF, ACTN1, ACTN4, PFN1, FSCN1, YWHAZ, YWHAH, YWHAZ, and PGAM1) were significantly increased in soft tissue 1 week post-extraction (Fig. 3C). The expression levels of both ATP5B (ATP synthase subunit beta, mitochondrial) mRNA and protein showed the same pattern of increase in soft tissue after tooth extraction (Table I and Fig. 3D). In addition, YWHAZ and ENO3 were dramatically increased in soft tissue 2 weeks post-extraction (Table I). This network (Fig. 3B) was centered on 14-3-3 protein zeta/delta (YWHAZ), which is known to be a key mediator in several signal transduction pathways. For example, YWHAZ has been implicated...
| Accession No. | Description                          | Gene     | Score (PLGS) | Sig. pep. | Seq. cov. (%) | Ratio in soft tissue | Ratio in bone |
|--------------|--------------------------------------|----------|--------------|-----------|--------------|----------------------|---------------|
| g118403912   | Pigment epithelium-derived factor    | SERPINF1 | 145.85       | -         | 54.5         | 2.11                 | 2.43          |
| g164318      | Albumin                              | ALB      | 3125.21      | +         | 36.0         | 0.67                 | 1.17          |
| g18623884     | Transferrin                          | TF       | 2644.29      | +         | 40.4         | 0.43                 | 1.93          |
| g136192       | Biglycan                             | BGN      | 517.40       | +         | 44.2         | 0.98                 | 1W            |
| g55742742     | Decorin precursor                    | DCN      | 138.02       | +         | 47.2         | 0.67                 | 1W            |
| g311273025    | Fibronectin isoform 3                | FN1      | 73.26        | +         | 25.0         | 1W                   | 1.77          |
| g19403768     | Lumican-like                         | LUM      | 265.45       | +         | 50.2         | 1.03                 | 1W            |
| g311251001    | Minecan-like                         | OCN      | 384.23       | +         | 45.6         | 0.49                 | 1W            |
| g311268153    | Periostin isoform 2                  | POSTN    | 201.13       | +         | 58.8         | 1.07                 | 1.51          |
| g31287975     | Collagen alpha-1(1) chain-like       | COL1A1   | 205.54       | -         | 70.6         | 0.79                 | 0.73          |
| g92020806     | Collagen alpha-1 chain, type VI      | COL6A1   | 155.36       | -         | 18.9''       | -                    | -             |
| g47523782     | Lactoferrin                          | LTF      | 213.50       | +         | 31.2         | 2.51                 | 2.10          |
| g312969753    | Alpha-2-HS-glycoprotein              | FETuin   | 199.23       | +         | 62.7''       | -                    | -             |
| g194033853    | Serpin A3-6                          | SERPINA3-6| 1384.85      | +         | 25.1         | 2.14                 | 0.91          |
| g312861517    | Serpin A3-8                          | SERPINA3-8| 908.67       | +         | 28.1         | 1.81                 | 0.81          |
| g47523270     | Alpha-1-antichymotrypsin 2           | SERPINA3-2| 826.48       | +         | 40.5         | 0.54                 | 1.34          |
| g121118       | Gestoln                              | GSN      | 186.12       | +         | 32.4         | 0.46                 | 1.72          |
| g47522736     | Hemopexin precursor                  | HPX      | 382.86       | +         | 61.0         | 0.64                 | 1.24          |
| g3259892      | MHC class I antigen                  | PA1      | 117.39       | +         | 7.5          | 1W                   | 0.37          |
| g47522844     | Complement C3                        | C3       | 278.39       | +         | 46.7         | 0.89                 | 1.73          |
| g311256213    | Alpha-2-macroglobulin                 | A2M      | 566.89       | +         | 56.9         | 1.49                 | 1.27          |
| g311256222    | Alpha-2-macroglobulin-like 1         | A2ML1    | 59.65        | +         | 42.9         | 1.58                 | 1W            |
| g311262435    | Protocaderhin-23                    | FGG      | 114.51       | -         | 29.9         | 1.40                 | 1W            |
| g29759175     | ATP synthase subunit alpha, mitochondrial | ATPA1    | 123.22       | -         | 55.7         | 2.26                 | 0.85          |
| g194037554    | ATP synthase subunit beta, mitochondrial | ATPB     | 135.72       | -         | 66.3         | 2.89                 | 0.73          |
| g311268235    | Prolin-1-like isoform 2              | PFN1     | 7212.33      | -         | 35.7         | 1W                   | 0.64          |
| g58926209     | Heat shock protein beta-1            | HSP27    | 1749.63      | -         | 65.2         | 1.32                 | 0.56          |
| g304365428    | Protein disulfide-isomerase A3       | PDA3     | 156.27       | +         | 31.7         | 1W                   | 1.51          |
| g304365440    | Protein disulfide-isomerase A6       | PDA6     | 921.87       | +         | 65.9         | 2.42                 | 1.39          |
| g47573262     | Transitional endoplasmic reticulum ATPase | VCP     | 71.13        | +         | 51.6         | 2.54                 | 0.73          |
| g311259673    | Histone H2A type 1-A-like            | HISTH2AA  | 3679.04      | -         | 56.3''       | -                    | -             |
| g194039766    | Histone H4-like                      | HISTH4A  | 3391.15      | -         | 17.5''       | -                    | -             |
| g147899784    | Elongation factor 1-alpha 1         | EEF1A1   | 523.60       | -         | 27.3         | 1W                   | 0.97          |
| g48374063     | Desmin                               | DES      | 657.78       | -         | 41.6         | 1W                   | 0.76          |
| g21431723     | Vimentin                             | VIM      | 141.50       | -         | 42.2         | 0.26                 | 1W            |
| g54020966     | Annexin A2                          | ANXA2    | 504.27       | -         | 62.8         | 2.13                 | 0.80          |
| g311262609    | Annexin A5-like, partial             | ANXA5    | 184.95       | -         | 60.8         | 1.98                 | 1.71          |
| g311261254    | Alpha-actinin-1-like                 | ACTN1    | 123.92       | -         | 62.2         | 3.42                 | 0.69          |
| g311257527    | Alpha-actinin-4-like                 | ACTN4    | 101.83       | -         | 58.6         | 2.26                 | 0.99          |
| g194044626    | 14-3-3 protein beta/alpha isoform 1  | YWHAB    | 163.34       | -         | 59.4         | 0.92                 | 2.04          |
in the initiation and progression of cancer and is overex-
pressed in various cancer types, including oral carcinoma (29, 30). Overexpression of YWHAZ is associated with anchorage-
independent growth and survival advantage under stress con-
ditions (31). The cellular functions of these networks were
ultimately associated with neurological disease, skeletal and
muscular disorders, and dermatological diseases and condi-
tions (supplemental Table S5). In addition, all six genes
(ATP5A1, ATP5B, EEF1A1, FGG, FN1, and GAPDH) in bone
tissue that have connections to targets in soft tissue were
increased after tooth extraction (Fig. 3B). Interestingly, at the
protein level these targets were not detected in unstimulated
bone (no tooth extraction) (Table I). Moreover, HIST1H4A,
which was detected only in bone, was dramatically increased
1 week post-extraction (ratios: 1W:No 2.25; 2W:1W 0.17
in Table I). All proteins involved in these networks were sig-
nificantly increased in both soft tissue and bone after tooth
extraction. We therefore suggest that these proteins might be
required for bone regeneration and healing.

Our data analysis also revealed a highly interconnected
group of 27 proteins involved in the dynamics of cellular
organization and maintenance that were differentially ex-
pressed in soft tissue and bone (Fig. 3A). This network was
mainly centered on targets and stimuli within extracellular
matrix (ECM), which is a composite of collagens and elastic
fibers embedded in a viscoelastic gel of proteoglycans, hya-
uronan, and assorted glycoproteins (32). These molecules
interact through entanglement, cross-linking, and charge-de-
pendent interactions to form bioactive polymers that, in part,
regulate the biomechanical properties of tissues and their
cellular phenotypes (33). The relative contributions of different
ECM molecules can vary with tissue type and exhibit mechan-
ical and chemical properties appropriate to each environment.
The actions of several targets in this group that were affected
by growth factors such as transforming growth factor β (TGF-β)
are thought to activate the ERK pathway. In soft
tissue, biglycan (BGN), decorin (DCN), and mimecan
were reduced or absent 2 weeks post-extraction (Table I). In
contrast, in bone tissue following tooth extraction, the expres-
sion of BGN, DCN, and mimecan was maintained at the
normal level (Table I), though lumican levels were somewhat
reduced, suggesting the importance of glycosylation for pro-
tein functionality. BGN, DCN, lumican, and mimecan are all
members of a small leucine-rich proteoglycan family and are

### Table I—Continued

| Gene ID | Protein Name                      | Protein Description            | Cell Type | Tissue Type 1 | Tissue Type 2 | Ratio Comparison | Notes                   |
|---------|-----------------------------------|--------------------------------|-----------|---------------|---------------|------------------|-------------------------|
| gi311251095 | 14-3-3 protein gamma-like         | YWHAZ                          | Soft Tissue | 86.25         | 43.31         | 1.96             | -                       |
| gi194048973 | 14-3-3 protein zeta/delta         | YWHAZ                          | Soft Tissue | 70.52         | 54.31         | 1.30             | -                       |
| gi311278800 | Neurofilament heavy polypeptide   | NEFH                           | Soft Tissue | 307.21        | 32.81         | 9.38             | -                       |
| gi143811428 | Neurofilament medium polypeptide  | NEFM                           | Soft Tissue | 314.52        | 22.22         | 14.14            | -                       |
| gi194041502 | Neurofilament medium polypeptide-1 | NFM1                          | Soft Tissue | 319.63        | 38.91         | 8.18             | -                       |
| gi7043802 | Rab GDP dissociation inhibitor beta | GDI2                          | Soft Tissue | 379.53        | 51.01         | 7.41             | -                       |
| gi31261519 | Serpin A3-3-like                  | SERPIN1                        | Soft Tissue | 288.30        | 53.31         | 5.40             | -                       |
| gi417185 | Leukocyte elastase inhibitor      | SERPIN1B1                      | Soft Tissue | 271.60        | 71.71         | 3.79             | -                       |
| gi31252249 | Macrophage-capping protein-like   | CAPG                           | Soft Tissue | 103.37        | 37.51         | 2.76             | -                       |
| gi13205498 | beta-enolase                     | ENO3                           | Soft Tissue | 113.38        | 62.21         | 1.82             | -                       |
| gi31250400 | Alpha-enolase-like               | ENO1                           | Soft Tissue | 190.60        | 66.41         | 2.80             | -                       |
| gi1364248 | Glucosephosphate isomerase       | GPI                            | Soft Tissue | 513.18        | 34.41         | 14.37            | -                       |
| gi65887 | Glyceraldehyde-3-phosphate dehydrogenase | GAPDH                    | Soft Tissue | 263.05        | 11.81         | 22.83            | -                       |
| gi47523764 | Peptidyl-prolyl cis-trans isomerase A | PPIA                      | Soft Tissue | 3625.21       | 56.11         | 65.84            | -                       |
| gi194041795 | Phosphoglycerate mutase 1-like   | PGAM1                          | Soft Tissue | 255.50        | 42.91         | 6.00             | -                       |
| gi194038728 | Pyruvate kinase isozymes M1/M2   | PKM2                           | Soft Tissue | 102.98        | 51.01         | 2.00             | -                       |
| gi162952052 | Transketolase                    | TKT                            | Soft Tissue | 87.33         | 43.51         | 1.97             | -                       |
| gi31263518 | Serpin H1                        | HSP47                          | Soft Tissue | 902.27        | 43.11         | 20.77            | -                       |
| gi31250274 | Peripherin                       | PRPH                           | Soft Tissue | 776.23        | 26.91         | 20.02            | -                       |
| gi194040624 | Plasmin-2 isozyme 1             | LCP1                           | Soft Tissue | 260.58        | 68.71         | 3.82             | -                       |
| gi225382135 | Fascin                           | FSCN1                          | Soft Tissue | 74.14         | 34.31         | 2.16             | -                       |
| gi194041957 | Alpha-intermixin-like            | INA                            | Soft Tissue | 315.32        | 19.01         | 16.63            | -                       |

* Predicted signal peptide in the protein sequence determined by Phobius.

b Sequence coverage (%) calculated in soft tissue.

c Ratio comparisons made to obtain the quantitative values 1W:No and 2W:1W.

d Predicted cellular localization analyzed by Cello (v 2.5) program.

e Sequence coverage (%) calculated in bone.
found in connective tissues such as bone, muscle, and blood vessels, as well as in the keratinocyte layer. They reportedly organize ECM assembly, bone formation and absorption, and osteogenesis (34, 35). Levels of α-2-macroglobulin protein were increased in soft tissue (ratios: 1W:No = 1.49; 2W:1W = 1.27; Table I) and bone (ratios: 1W:No, detected only in 1W;
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2W:1W = 0.72; Table I) after tooth extraction, and increased levels of transcript were also detected in soft tissue (Fig. 3D). It was previously reported that α-2-macroglobulin exerts effects on collagen fiber organization at the healing tendon–bone interface (36, 37) and that α-2-macroglobulin mRNA, which is known to share vascular, osteogenic, and cartilage functions relevant for avascular necrosis of the femoral head, is overexpressed in rats with steroid-induced avascular necrosis of the femoral head (37). Interestingly, serpin peptidase inhibitor, clade F, member 1 (SERPINF1) was continuously increased in soft tissue after tooth extraction at both protein and mRNA levels (Table I and Fig. 3D). SERPINF1, also known as pigment epithelium-derived factor, was originally identified as a potent inhibitor of angiogenesis, the proliferation and migration of endothelial cells, retinal vascular permeability, and tumor activity (38). SERPINF1 expression changes with the progression of various tumor types (39), which suggests that SERPINF1 may direct cellular fate. It was also recently reported that a frameshift mutation in exon 4 of the SERPINF1 gene that reduced expression of the transcription/translation product and causes progressively deforming osteogenesis imperfecta is likely a key factor in bone deposition and remodeling (40).

The expression of fibronectin 1 (FN1) protein and mRNA was enhanced in both soft tissue and bone after tooth extraction. Cellular fibronectin has numerous functions involved in cell adhesion, growth, migration, and differentiation (41). In particular, fibronectin profoundly affects wound healing, including the formation of a proper substrate for cell migration and growth during the development and organization of granulation tissue, as well as synthesis and remodeling of the connective tissue matrix (42, 43). In addition, RUNX2 appears to up-regulate FN1 in osteoblasts in vitro (44) and to be required for osteoblast differentiation and mineralization (45, 46). These proteins have been shown to modulate multiple processes related to ECM function, including the interaction of cytoskeletal and ECM constituents. Our network analysis highlighted the modification and reconstruction of ECM and communication between gingival soft tissue and alveolar bone for bone healing.

Interaction of ATP5B and FN1 with Cellular Functionality—Among the 62 identified proteins, we examined the protein interactions involving ATP5B and FN1 in cellular functionality in greater detail. We observed the predicted association between various proteins and ATP5B or FN1, based on their observed patterns of simultaneous expression (supplemental Fig. S1 and supplemental Table S6). Consistent with the MS result (ratios: 1W:No = 2.89; 2W:1W = 0.73 in Table I), ATP5B expression was increased in soft tissue after tooth extraction (Fig. 4A). In humans, ATP5B is involved with molecular transport, nucleic acid metabolism, and small molecule biochemistry, along with ATP5A1, PON1, PON2, YWHAZ, PGAM1, and GDI2 (left-hand side of Fig. 4A). This network is central to the interaction between ATP5B and YWHAZ. Previously, MS was used to show that the levels of these two proteins are lower in osteosarcoma than in benign bone tumors, including osteoblastoma, chondroblastoma, and giant cell tumors of bone (47). In addition, levels of ATP5B and YWHAZ mRNA are unstable during cutaneous wound healing (48). In other species, including M. musculus, S. cerevisiae, and C. elegans, ATP5B has been connected to nucleic acid metabolism, small molecule biochemistry, and neurological disease, along with GAPDH, ENO1, ENO3, PDI1, ATP5A1, GDI2, TKT, and EEF1A1 (right-hand side of Fig. 4B). More specifically, the primary stimulus for this connection was focused on neuronal disorders caused by aberrant posttranslational modifications, including ubiquitination and proteolytic cleavage (49). These proteins are thought to play a collateral role in bone regeneration via a network linking protein stability and mitochondrial signaling.

IHC showed that FN1 levels were increased 1 week after tooth extraction and had declined by 2 weeks post-extraction (Fig. 4B). In vitro, FN1 up-regulates mineralized osteoblasts relative to nonmineralized osteoblasts (46). As expected, within the extracellular space, FN1 was coordinated with cellular assembly and organization and cellular maintenance, along with DCN, COL1A1, BGN, COL6A1, and POSTN. The possible link between these proteins is TGF-β, which is crucial for connective tissue regeneration and bone remodeling (50, 51). TGF-β affects osteoblast differentiation and bone formation (52, 53) and increases transcription of osteoblast differentiation markers and alkaline phosphatase activity in murine bone marrow stromal cells (54). FN1, which is a TGF-β target gene, might be a trigger factor induced during bone healing and regeneration, as it has interactions involving cellular adhesion and migration processes, including wound healing, blood coagulation, host defenses, and metastasis. Taken together, our data show that ATP5B and FN1 are differentially expressed in soft tissue and bone after tooth extraction, which suggests their participation in the transduction of bone regeneration signals.
DISCUSSION

Our aims in this study were to compare the patterns of protein expression in gingival soft tissue and alveolar bone following tooth extraction and to identify specific targets for bone regeneration (hard tissue) within soft tissue. Quantitative proteomics data represent an important source that can be used to expand our understanding of the molecular basis of the communication between soft tissue and hard tissue during bone healing.

Bone is a dynamic and heterogeneous tissue and is continually being remodeled in physiological circumstances through the bone-forming activities of osteoblasts and the bone-resorption activities of osteoclasts. Bone mass and turnover are maintained through the coordinated activities of these two cell types under the regulation of numerous systemic and local factors (55, 56). Osteoblasts are bone-forming cells that synthesize an ECM and then participate in the mineralization of that matrix (46). Similar repair and regener-
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The inverse correlation between osteoclast survival and bone resorption regulated by ATP6B provides new insight into the molecular mechanisms regulating bone homeostasis.

The dynamic assembly of the FN1 matrix is cell specific and a key event regulating cell adhesion, migration, and differentiation (87). Cell adhesion to ECM is essential for the development, maintenance, and remodeling of osseous tissues (88). Adhesive interactions with ECM components, including fibronectin and type I collagen, play critical roles in osteoblast survival, proliferation, and differentiation and in matrix mineralization and bone formation (89). For that reason, we will next focus on analyzing these ECM regulatory proteins in more detail using more refined bone-healing models and bone grafting. We anticipate that our findings will shed new light on the interaction between gingival soft tissue and alveolar bone and on the cellular signaling underlying bone remodeling.

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