Oral fibroblast expression of wound inducible transcript-3.0 (wit3.0) accelerates the collagen gel contraction in vitro.

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Summary

Wounds of the oral mucosa show faster closure with less scar formation than skin wounds in other areas. A differentially-expressed cDNA, *wound inducible transcript-3.0 (wit3.0)*, was isolated from oral mucosal wound in rats (Sukotjo et al., J Den Res 81:229-235, 2002). The purpose of this study was to characterize the *wit3.0* gene structure and the function of its deduced peptide. Human and rat genome databases revealed that the gene for *wit3.0* was located in human chromosome 12p11.23 and rat chromosome 4q44. Its human and rat gene structures were well conserved, composed of 7 exons spread over 20 kb. Exon 5 was alternatively spliced generating two transcripts encoding deduced peptides of 215 and 253 amino-acids (*wit3.0α* and *wit3.0β*, respectively). The protein families database of alignments (Pfam) analysis suggested the *wit3.0* peptide sequence shared similarity with a portion of the myosin II coiled-coil domain consensus sequence. Fibroblasts isolated from the rat oral wound up-regulated *wit3.0* expression and exhibited greater ability to contract collagen gel *in vitro* than fibroblasts isolated from untreated oral mucosa/gingiva. NIH3T3 and rat oral fibroblasts transfected with expression vector containing the coding sequences of *wit3.0α* or *wit3.0β* increased *in vitro* collagen gel contraction. When treated with TGFβ-1, NIH3T3 fibroblast expression of *wit3.0* showed no significant change, while alpha smooth muscle actin was increased in a dose-dependent manner. These data suggest that there may be a novel wound healing pathway involving *wit3.0* underlying the favorable early wound closure characteristics of oral mucosa.
Introduction

Tooth extraction is one of the most frequently prescribed oral surgery (1). The surgical removal of tooth creates a large open wound, which heals generally without complication (2). Characteristically, the oral mucosa open wound closes rapidly primarily due to the spontaneous approximation of gingival wound margins, often without the aid of surgical suturing (Fig. 1). Oral mucosa and skin wounds undergo the similar healing sequence, including hemostasis, inflammation, granulation tissue formation and remodeling of the connective tissue matrix. However, clinical observations and experimental animal studies consistently indicate that the extent of granulation and scar formation in oral mucosa is generally small, and wound healing in the oral mucosa demonstrate the better outcome as compared to the equivalent wound in skin (3-5). Oral mucosa and gingiva are composed of a thin keratinocyte layer with underlying a highly vascularized connective tissue (6). Although a number of investigations had attempted to explain the healing differences between oral mucosa and skin (7-9), the distinctive mechanism involved in oral mucosal wound healing remains to be elucidated.

Isolated oral fibroblasts have been shown to contract the collagen gel in vitro at a faster rate than skin fibroblasts (5,10-12). These data suggest that oral fibroblasts possess distinctive characteristics promoting accelerated wound closure. We recently isolated the overlapping full-length cDNAs encoding a differentially-expressed gene, wound inducible transcript-3.0 (wit3.0) from a cDNA library of rat gingiva tissue undergoing wound healing after tooth extraction. We also found that appearing to generate from the wit3.0 gene, two alternative transcripts presented; encoding 215 (wit3.0α) and 253 (wit3.0β) amino-acid long peptides. In situ hybridization has revealed that the fibroblasts localized in the oral wound were the cellular source of wit3.0 (13).
A Basic Local Alignment Search Tool (BLAST) search matched \textit{wit3.0} with human EST, FLJ10672; however, the identification and potential function of \textit{wit3.0} are still unknown. This study aimed to characterize the \textit{wit3.0} gene structure and to identify the potential function of its deduced peptide. The results suggest that there may be a novel wound healing pathway involving \textit{wit3.0} facilitating fibroblast-derived oral wound contraction that may contribute to the favorable early wound-closure characteristics of oral mucosa.
Experimental Procedures

Rat tooth extraction wound healing model

Experimental wound in oral mucosa was created in rats by unilateral maxillary molar extraction (14,15). Forty-day-old Sprague-Dawley rats were anesthetized with 2% isoflurane inhalation. The gingival tissue firmly attached to the maxillary molars was carefully dissociated by a dental explorer and the maxillary first, second and third molars were extracted from the left jaw. The wound healing tissue and the contra-lateral untreated oral mucosa/gingival tissue were harvested at the predetermined healing time as described below.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) studies on steady state mRNA levels of the extracellular-matrix molecules, wound related molecules and wit3.0 during wound healing

The oral mucosa wound specimen, as well as untreated contra-lateral gingival tissue was harvested at postsurgery day 4 and 7. Each tissue specimen was homogenized separately and total RNA was extracted by the guanidium isothiocyanate method (Trizol, Gibco Life Technologies, Grand Island, NY). After the DNase treatment, 1 μg of total RNA sample was used to synthesize cDNA using random hexamer primer (Clontech, Palo Alto, CA). Steady state mRNA levels of the following extracellular matrix (ECM) molecules and wound-related peptides were examined using RT-PCR: type I collagen alpha 1 chain (col1a1), type III collagen alpha 1 chain (col3a1), type XII collagen alpha 1 chain isoforms A, B, 1 and 2 (col12a1A, col12a1B, col12a1-1, and col12a1-2, respectively), TGF-β1, α-smooth muscle actin (α-SM actin) as well as wit3.0α and wit3.0β. Primer sequences and PCR conditions achieving the exponential amplification for each target mRNA molecule are summarized in Table 1. During the
preliminary experiments, PCR conditions were determined to represent the exponential amplification cycle for each target molecule. Throughout the experiment GAPDH was used as the normalization control.

In vitro gel contraction assay of fibroblasts from wounded and untreated rat oral mucosa/gingiva

Fibroblasts from healing tooth extraction wounds and untreated gingiva were harvested at postsurgery day 7. The tissue was minced into ~ 2 mm³ under phosphate buffered saline, pH 7.4 (PBS), then rinsed twice with PBS. Minced pieces of the tissue were planted onto the surface of 10-mm cell culture dishes. After 1 hour of incubation at 37 °C, 10 ml of growth medium: Dulbecco’s Modified Eagle’s Medium (DMEM) and 10% Fetal Bovine Serum (FBS), with 0.25 µg/mL Amphotericin B, 100 U/mL Penicillin, and 100 µg/mL Streptomycin antibiotics, was added. The fibroblasts were obtained by trypsinization of the primary outgrowth of cells. Isolated primary cells were cultured under 37 °C, 5 % CO₂, and 80% humidity condition. Growth medium was continually replenished every 4 days. The cells were routinely passaged using 0.05 % trypsin in PBS containing 0.53 mM EDTA. Cells used for the experiments were collected from between the 4th and 10th passages. The steady state level of wit3.0 mRNA was examined by RT-PCR as described above.

Collagen gels were casted in 6 well plates from type I collagen/ DMEM solution composed of 5 parts of bovine skin collagen type I (Vitrogen 100, Cohesion Corp., Palo Alto, CA), 2 parts of 5 X DMEM containing Hepes and gentamicin, 1 part of NaOH (0.142 M), 5 parts of FBS, and 1.5 parts of PBS (16). The gels were in liquid form at 4°C, and solidified at 37°C. Primary
fibroblasts were seeded into the collagen gel (1.2 X10^5 cells/well) and incubated at 37°C, humidity of 80%, and CO₂ level of 5%. Fibroblasts/gel complex contraction was monitored by standardized photography every 10 hours for 120 hours.

The area of fibroblast/gel complex in digitized photographs was measured using Image Pro Plus software (Media Cybernetics, Maryland, USA). The ratio of collagen gel area against the culture well area was calculated at each measurement point. Multiple data sets of different groups and different time points were analyzed by repeated measures analysis of variants (ANOVA), or two-way ANOVA at a 5% level.

**In silico search for human, rat and mouse genome databases and the protein families database of alignments (Pfam)**

The nucleotide sequence of rat wit3.0 was used to search chromosomal assignment and gene structure in silico using the public human, rat and mouse genome databases. The genomic sequences were further compared with the cDNA sequences and the exon structure was determined. The deduced peptide sequences of *wit3.0α* and *wit3.0β* were submitted to the online protein families database of alignment (Pfam) search (16,17), for a search of the available consensus functional domains. The *wit3.0* peptide sequence and the candidate sequences were compared by the protein BLAST search.

**Cellular localization of wit3.0-FLAG fusion peptide in NIH3T3 fibroblastic cells**

Expression vectors containing a CMV promoter leading fusion peptides of FLAG epitope (Sigma Chemical, St. Luis, MO) and *wit3.0α* or *wit3.0β* coding sequences were constructed. NIH3T3
fibroblastic cells were transfected with the \textit{wit3.0}-FLAG fusion peptide expression vectors using lipofectamine (Lipofectamine 2000, Gibco BRL, Grand Island, NY) at 70\% confluency. Transfected cells were cultured at 37°C in DMEM (Gibco BRL, Grand Island, NY), supplemented with 10\% FBS and 100U/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B, under 5\% CO₂ conditions for 24 to 48 hours.

Transfected NIH3T3 fibroblastic cells were centrifuged at 500 x g for 2-3 minutes; nuclear and cytoplasmic extracts were separately collected using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL) using the manufacturer’s protocol. The extracts in Laemli buffer containing b-Mercaptoethanol were subjected to 4-20\% SDS gel electrophoresis. The transferred Western blot was examined with M2 anti-FLAG monoclonal antibody using HSP89 monoclonal antibody as a positive cytoplasmic protein control.

For fluorescent cytology experiments, NIH3T3 fibroblastic cells were cultured on Permanox Chamber Slide (Lab Tek, Nalgen Nunc Int., Rochester, NY). Transfection of NIH3T3 fibroblastic cells using recombinant expression vector containing coding sequences of \textit{wit3.0α}, \textit{wit3.0β} and bacterial alkaline phophatase (BAP: positive control) was performed as previously described. After 24 to 48 hours, cells were washed with TBS/CA (50 mM Tris, 150 mM NaCL, pH 7.4, containing 1mM Calcium Chloride) and fixed with freshly prepared 1:1 mix of acetone/methanol for 1 minute. Ten micrograms/ml of M5 anti-FLAG monoclonal antibody was used as a primary antibody to recognize the FLAG epitope. After 1 hour primary antibody incubation, the chambers were washed five times with TBS/CA. Texas-Red, fluorescin-conjugated antibody (Molecular Probes, Oregon) was used as a secondary antibody. Sytox green
(Molecular Probes, Oregon) was used to stain the cell’s nucleus and a confocal laser scanning microscope was used to determine the terminal localization of the \textit{wit3.0} translated peptides.

\textit{In vitro gel contraction assay for NIH 3T3 fibroblastic cells and rat gingival fibroblasts transfectected with \textit{wit3.0} expression vectors}

To test the hypothesis that \textit{wit3.0} participates in facilitating fibroblast-derived wound contraction, NIH3T3 fibroblastic cells transfected with the expression vectors containing \textit{wit3.0\textalpha-FLAG}, \textit{wit3.0\textbeta-FLAG}, and bacterial alkaline phosphatase-FLAG fusion peptides were subjected to the \textit{in vitro} collagen gel contraction assay. The areas of collagen gel of the following four groups were compared: NIH3T3 + \textit{wit3.0\textalpha-FLAG}, NIH3T3 + \textit{wit3.0\textbeta-FLAG}, NIH3T3 + BAP-FLAG (transfection control) and NIH3T3 cells alone (no transfection control).

Anti-sense oligonucleotide treatment was performed to validate the effect of \textit{wit3.0} on gel contraction. Anti-sense oligonucleotide: 5’-CTGAATGGTGCAGCTCAT-3’ and Sense oligonucleotide: 5’-ATGAGCTGCACCATTCAG-3’ were dissolved in DMEM culture medium into 100\(\mu\text{M}\) stock solution and sterilized by filtration through 0.2\(\mu\text{M}\) cellulose acetate filter.

Serum free medium containing 2\(\mu\text{M}\) oligonucleotide was mixed with lipofectamine and incubated for 20 min at room temperature. The mixture was later added to the NIH3T3 fibroblastic cells that had previously received one of each expression vectors containing \textit{wit3.0\textalpha-FLAG}, \textit{wit3.0\textbeta-FLAG} or BAP-FLAG fusion peptide. Cells were incubated for 4 hours without serum prior to addition of FBS. Subsequent, the cells were divided into two groups, the first group of was further incubated for 24 hours at 37\(^\circ\text{C}\) and subjected for Western blot assay using M2 anti-FLAG monoclonal antibody. The second group was mixed with bovine type I collagen.
gel solution (Vitrogen 100, Cohesion Corp, Palo Alto, CA) as described above to a concentration of 1 x 10^5 cells/ml/well. Non-transfected cells serve as an untreated control in both groups. Consistent volume of collagen gel was used throughout the experiment. Solidified gel was released from the well after 2 hours of incubation. Standardized photography was used to monitor the longitudinal gel contraction.

In a separate experiment, rat gingival fibroblasts were transfected with each of expression vectors containing wit3.0α-FLAG or wit3.0β-FLAG, or both simultaneously. The expression levels of wit3.0 mRNAs were confirmed by RT-PCR. The transfected and untreated rat gingival fibroblasts as well as rat oral wound fibroblasts were subjected to in vitro collagen contraction assay as described above.

*Transforming Growth Factor-β1 (TGF-β1) treatment to NIH3T3 fibroblastic cells*

NIH3T3 fibroblastic cells were cultured in a 6-well plate at a cell density of 1X10^4 cells/ cm². The cells were starved for 12 hours in DMEM supplemented with 0.5% FBS and 1X Amphotericin B and Penicillin (3ml/well). After 12 hours, 0.1 ng/ml or 5.0 ng/ml of TGF-β1 was added to the culture medium. No TGF-β1 addition was used as a control. The cells were cultured at 37°C, humidity of 80%, and 5% CO₂ level for an additional 18 hours until total RNA from the cells was extracted using the Trizol method. α-SM actin and wit3.0 expressions were examined by RT-PCR. The housekeeping gene, β-actin, was used to standardize the gene expression level. Primer sequences and PCR conditions representing experiments performed in triplicate are listed in Table 1.
Results

Oral mucosa wound healing profile

Rapid wound healing as shown in the rat oral wound model was reinforced in our studies. Following tooth extraction, by day 4 of wound healing, granulation tissue, through which thin epithelial cell layers migrate (Fig. 1C, arrowheads), was apparent. By day 7, the wound closure progresses by approximation of the wound margins (Fig. 1D). During early wound healing stage, TGFβ-1 and α-SM actin mRNAs increased moderately, whereas synthesis of both wit3.0α and wit3.0β mRNAs was robust. Expression of type I collagen mRNA during first week remained at the baseline level, while synthesis of type III, type XIIB-1 and XIIB-2 collagens increased (Fig. 1E).

Fibroblasts isolated from day 7 wound healing tissue maintained the high level of wit3.0 expression even after 10 passages. Both wound fibroblasts and untreated gingival fibroblast controls exhibited more than 95 % contraction during the first 15 to 20 hours of incubation in the in vitro collagen gel contraction assay (Fig. 1F). Wound fibroblasts/gel complex showed significantly greater intensity of contraction than untreated control fibroblasts/gel complex (P<0.05). Although the contraction rate of both leveled off after 20 hours, fibroblasts from the wounded tissue exhibited greater overall gel contraction (42% ± 7.3 of its original area) compared with that of untreated gingival fibroblasts (76% ± 9.6) as shown in Fig. 1F.

In silico evaluation of the wit3.0 gene and peptide structures

Matches in the online genome databases located the nucleotide sequence of wit3.0 within human chromosome 12p11.23 and rat chromosome 4q44 (Fig. 2A). The genomic DNA structures of
human and rat \textit{wit3.0} were highly conserved and encoded by 7 exons spread over 20 kbp (Fig. 2B). The deduced peptide sequence was encoded by a part of exon 2, exons 3-6 and a part of exon 7. Exon 1 encoded the 5’ untranslated region; exon 7, the large 3’ untranslated region. Exon 5 encoded the in-frame insertion sequence found in \textit{wit3.0}β, but not in \textit{wit3.0}α (Figs. 2B and 2C).

Rat \textit{wit3.0} deduced peptide sequence was searched in BLAST for homologous sequences. Results showed matches with myosin heavy chain among Oryctolagus cuniculus (44% positive amino acid match including 22% identical match), Drosophila melanogaster (42% positive amino acid match including 21% identical match), and Argopecten irradians (43% positive amino acid match including 24% identical match). The 228 amino acids that match with skeletal muscle myosin heavy chain belong to a conserved domain: pfam01576.8 (35% positive amino acid match including 15% identical match). With a consensus sequence of 860 amino acids, pfam 01576.8 myosin tail family consists of the coiled-coil myosin heavy chain tail region (Fig. 3). In addition, \textit{wit3.0} matched with pfam COG4372 (499 amino acids long), which is an uncharacterized protein conserved in bacteria with the myosin-like domain (data not shown).

\textit{Intracellular Peptide Localization}

NIH3T3 fibroblastic cells over-expressing the \textit{wit3.0}-FLAG fusion peptide showed that both \textit{wit3.0}α-FLAG and \textit{wit3.0}β-FLAG peptides were detected in the cytoplasmic fraction by Western blot (Fig. 4A and 4B, respectively). \textit{wit3.0}α and \textit{wit3.0}β-FLAG peptides were demonstrated as the single band of 40 kDa and 43 kDa, respectively, with or without the β-mercaptoethanol
treatment. Confocal laser scanning microscopy confirmed that wit3.0α and wit3.0β localized in the cytoplasm of transfected fibroblasts (Fig. 4C and 4D, respectively).

The effect of wit3.0 on collagen gel contraction

Gel contraction rate in NIH3T3 fibroblastic cells over-expressing wit3.0α or wit3.0β was significantly higher than the control groups (p<0.05) as shown in Table 2. The BAP transfection control and non-transfected control groups showed no differences in gel area size at each time point. The anti-sense oligonucleotide designed to block wit3.0β mRNA partially inhibited the wit3.0β-FLAG peptide synthesis. The accelerated collagen gel contraction in the wit3.0β transfection group was partially but significantly blocked by the anti-sense treatment (p<0.05) (Fig. 5A). Treatment with the sense oligonucleotide also decreased the wit3.0β-FLAG peptide synthesis and proportionately decreased the accelerated collagen gel contraction. The effect of wit3.0 on the collagen contraction lasted up to 15-20 hours. Similarly, the rat gingival fibroblasts over-expressing wit3.0α or wit3.0β peptides exhibited the increased collagen gel contraction as compared with the untransfected gingival fibroblast control (p<0.05). The gingival fibroblasts received both wit3.0α and wit3.0β expression vectors demonstrated more increased collagen gel contraction as compared with the untransfected gingival fibroblast control (p<0.01); however, the contraction rates of these transfected gingival fibroblast groups did not reach the collagen gel contraction rate of wound fibroblasts (Fig. 5B).

The effect of TGF-β1 on wit3.0 expression

The expression of α-SM actin mRNA in NIH3T3 fibroblastic cells increased 1.6 fold with 0.1 ng/ml and 1.8 fold with 5.0 ng/ml of TGF-β1, compared with the untreated control as shown in
Fig. 5C. NIH3T3 fibroblasts expressed both \textit{wit3.0} and \textit{wit3.0}$\beta$ mRNAs at baseline level. When treated with TGF-$\beta$1 (0.1 and 5.0 ng/ml), NIH3T3 fibroblasts showed non-significant increase in \textit{wit3.0}$\alpha$ and \textit{wit3.0}$\beta$ mRNA levels (Fig. 5D). Factorial ANOVA analysis revealed that only $\alpha$-SM actin increased significantly with TGF-$\beta$1 treatment ($p < 0.05$). The steady state mRNA levels of \textit{wit3.0}$\alpha$ and \textit{wit3.0}$\beta$ were not significantly affected by the TGF-$\beta$1 treatment.
**Discussion**

As shown, rapid wound closure by the approximation of wound margins in the rat oral musal wound by tooth extraction (Fig. 1A-D was observed, along with accelerated collagen gel contraction during the initial 15 to 20-hour period (Fig. 1F). The inflammatory response to the injury in oral mucosa has been shown less than in the equivalent dermal wound (9), and the expression of cytokines is relatively minimal in oral wound tissue during the healing period compared to other tissues (5,10-12,18). It has been postulated that saliva contains these cytokines and supplies them to oral wounds (19,20). The RT-PCR analysis in the present study generally support that the transcriptional response to the tooth extraction wound was mild to moderate among the genes examined. Therefore, it was distinctively noted that the steady state mRNA level of *wit3.0* increased the most from an initial minimal state to 40 fold within 4 days and over 50 fold after one week of healing.

Utilizing the genomic DNA sequence databases of humans and other animals recently available, the chromosomal location of *wit3.0* was determined to be 12p11.23 in the human and 4q44 in the rat. The mouse database indicated a *wit3.0* match, but the chromosomal location has not yet been determined. The Online Mendelian Inheritance in Man (OMIM) database (21,22) for known inheritable diseases linked to the 12p11.23 allele did not reveal *wit3.0* linkage to any recorded diseases.

The overlapping rat *wit3.0* cDNAs of 2746 bp suggest a small open reading frame of 645 bp (13). The online genome database search showed that the open reading frame was encoded from exon 2 through exon 7 in both humans and rats. The disproportionately large 3’ untranslated
region was encoded in the last exon 7. Based on the complete agreement between cDNA and genomic DNA exon sequences, as well as the highly conserved gene structure, we concluded that the coding sequence of \textit{wit3.0} might translate to a functional protein. Furthermore, our new data clearly show that alternative splicing of exon 5 accounts for the previously reported two different transcripts, \textit{wit3.0}\textalpha and \textit{wit3.0}\textbeta (13).

The \textit{wit3.0} deduced peptide was correlated with the Pfam consensus sequence motif of myosin heavy chain II coiled-coil domain (Fig. 3). Myosin heavy chain II is a multidomain protein important for both cellular structure and contraction (23,24). Myosin heavy chain II molecules are dimerized through the coiled-coil \(\alpha\)-helix and provide the phosphorylation site. Thus, the coiled-coil domain is thought to regulate the myosin’s ability to produce force (25-27). The peptide sequence of \textit{wit3.0}\textbeta suggests a potential \(\alpha\)-helix structure (Fig. 2C). The \textit{wit3.0-FLAG} peptide examined on the SDS-PAGE Western blot appeared as a single peptide (Fig. 4A and 4B); however, because the myosin dimer is formed through ionic bonding, the possible molecular interaction by \textit{wit3.0} remains to be examined.

Our studies localized both \textit{wit3.0}\textalpha and \textit{wit3.0}\textbeta to cytoplasmic sites in the fibroblasts we studied. Together with the possible structural relationship with the myosin heavy chain II, which plays a regulatory role in the myofilament/cytoskeleton complex, we postulated that \textit{ wit3.0} may participate in the fibroblast-derived wound contraction. Tested in our \textit{in vitro} collagen gel contraction assay, fibroblasts over-expressing \textit{wit3.0}\textalpha or \textit{wit3.0}\textbeta peptides contracted the collagen gel at the faster rate than controls. The accelerated \textit{in vitro} gel contraction was temporally limited to the first 15 to 20-hour period. Because the presence of \textit{wit3.0-FLAG} fusion peptides
was confirmed by Western blot after the collagen contraction assay (data not shown), the change in the collagen contraction rate only during the initial period is unlikely due to depletion of wit3.0 expression vectors. There is a considerable similarity in the gel contraction profile between transfected fibroblasts over-expressing wit3.0 and oral wound fibroblasts, which continued to upregulate the endogenous wit3.0 expression. Thus, we speculated that the wit3.0’s contribution to wound contraction might require unidentified co-factors or partner molecules that are not simulated in the in vitro experiments.

Early wound contraction, typically characterized by actively proliferating, migrating epithelial cells and loose connective tissue ECM, is an essential initial healing process, which establishes the epithelial integrity in the dermal and mucosal open wound. Clinical observations suggest that migration of fibroblasts into and through the ECM during the initial phase of wound healing, prior to the expression of α-SM actin, appears to be a fundamental component of wound contraction (28). Due to the greater compliance of immature connective tissue, early wound contraction does not result in generating tensile stress. The wound repair process continues to precipitate ECM and the wound connective tissue increasingly becomes rigid. The inflexible ECM combined with a group of cytokines stimulates the differentiation of myofibroblasts expressing α-SM actin (29,30) that are more prevalent in the late wound repair tissue. The findings in our studies supported that TGFβ stimulated the α-SM actin expression. Other studies showed TGFβ has a capacity to accelerate the contraction rate of NIH3T3, BHK-21 cell lines, and human foreskin fibroblast cultured in collagen gel (31), which has been believed due to the increased synthesis of α-SM actin.
 Whereas the myofibroblast-mediated wound contraction mechanism has been well investigated, the mechanism responsible for the initial wound contraction is poorly understood. The involvement of \( \text{wit3.0} \) in the initial wound contraction may shed the light to new investigations on this important wound healing process.

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Figure Legend

Fig.1. Tooth extraction wound healing in humans and rats.

(A) Clinical picture of typical tooth extraction socket immediately after tooth extraction. (B) The same patient examined one week after the tooth extraction. Arrows show the distance between wound margins of the extraction socket. (C,D) H&E Stained histology sections at 10X magnification of rat frontal gingiva: (C) 4 days after tooth extraction; and (D) 7 days after tooth extraction. (A, B, C and D) The double-headed arrows show the distance between wound margins of the extraction socket. Arrowheads indicate the epithelial migration into the newly formed granulation tissue at the wound site. (E) Comparison of the mRNA expression profiles among the genes encoding TGF-β1, α-smooth muscle actin, wit-3.0α, wit-3.0β, α1(I) collagen, α1(III) collagen, α1(XII) collagen isoforms B,1 and 2 (normalized by GAPDH) in gingiva tissue between dormant and healing stages. (F) In vitro collagen-gel contraction in rat untreated gingival fibroblasts and wound fibroblasts at cell concentrations of 1.2 X10^5 cells/ well. The inset figure indicates wit-3.0 mRNA expression in untreated fibroblasts (lane 1) and wound fibroblasts (lane 2).

Fig.2. In silico study of wit3.0.

(A) The location of wit3.0 in a Human and Rat chromosomes. (B) Human and Rat gene structures. There are 7 exons in both human and rat. The start codon lies in the exon 2, and stop codon lies in exon 7. (C) Hydrophilicity diagrams of deduced peptide structure of wit3.0α and wit3.0β. An extra domain encoded by exon 5 is highlighted as a box in wit3.0β. The locations of 2 cysteines are indicated as letter C in wit3.0α; wit3.0β contains an additional cysteine in the exon 5 region as indicated.
Fig.3. Peptide sequence comparison between wit3.0β and myosin heavy chain II.

The deduced peptide sequence of rat wit3.0β (row 1) is aligned with the Pfam consensus sequence of myosin heavy chain II tail domain (row 2). The equivalent domains of myosin heavy chain from fruit fly (row 3), rabbit (row 4) and scallop (row 5) are also shown. The starting amino acid numbers are listed on the left. The identical amino acid matches are indicated by dark gray boxes, and the positive amino acid matches are indicated by light gray boxes.

Fig.4. Western blot and immunocytology of the wit-3.0-FLAG fusion peptide.

(A) The Western blot demonstrates cytoplasmic fraction(c), but not the nuclear fraction (n) of NIH3T3 fibroblasts exhibits the presence of wit3.0α-FLAG peptide after 24 hr, 48 hr, and 72 hr of expression vector transfection. (B) Similarly, wit3.0β-FLAG peptide appears terminally-localized in the cytoplasmic fraction. HSP86 was used as a positive control (cytoplasmic-specific protein). (C,D) Confocal laser-scanning microscopy of immunocytological localization of the wit3.0-FLAG peptide (rhodamine) showing cytoplasmic localization of wit3.0α(C) and wit3.0β(D). Sytox Green fluorescent dye was used to detect the nucleus.

Fig.5. Collagen contraction by NIH3T3 fibroblasts or primary fibroblast by over-expressing wit3.0 and the effect of TGF-β1 on wit3.0 expression.

(A) Four groups of NIH3T3 fibroblasts (1) transfected with wit3.0β-FLAG expression vector, (2) transfected with wit3.0β-FLAG expression vector and sense oligonucleotide, (3) transfected with wit3.0β-FLAG expression vector and antisense oligonucleotide, and (4) untreated were seeded in
the collagen gel. The accelerated collagen gel contraction by \textit{wit3.0}\textit{β} was partially blocked by the antisense treatment. The inset figure depicts \textit{wit3.0}\textit{β}-FLAG peptide of the non-transfected group (lane 1), the \textit{wit3.0}\textit{β}+sense oligonucleotide treated group (lane 2), and the \textit{wit3.0}\textit{β} +antisense treated group (lane 3). (B) Four groups of unwounded primary gingival fibroblasts (1) transfected with \textit{wit3.0}\textit{α}-FLAG expression vector, (2) transfected with \textit{wit3.0}\textit{β}-FLAG expression vector,(3) transfected with both \textit{wit3.0}\textit{α} and \textit{β}-FLAG expression vectors, (4) untreated and one group of wounded primary gingival fibroblasts untreated were seeded in the collagen gel. The inset figure depicts the gene expression level of \textit{wit3.0}\textit{α} and \textit{wit3.0}\textit{β} in the following groups: wounded fibroblasts (lane 1), unwounded fibroblasts (lane 2), unwounded fibroblasts +\textit{wit3.0}\textit{α} (lane 3), unwounded fibroblasts +\textit{wit3.0}\textit{β} (lane 4), and unwounded fibroblasts +\textit{wit3.0}\textit{α} and \textit{wit3.0}\textit{β} (lane 5). (C) Steady state mRNA level of \textit{α}-smooth muscle actin in NIH3T3 fibroblasts treated with 0, 0.1, and 0.5 ng/ml TGF-\textit{β1}. \textit{α}-smooth muscle actin mRNA increased 1.6 fold with 0.1 ng/ml and 1.8 fold with 0.5 ng/ml TGF-\textit{β1}. (D) Steady state levels \textit{wit-3.0}\textit{α} (white bars) and \textit{wit-3.0}\textit{β} (black bars) were not affected by the TGF-\textit{β1} treatment. \textit{wit3.0}\textit{α} and \textit{β} mRNA increased 1.12 and 1.04 fold with 0.1 ng/ml and 1.23 and 1.13 fold with 0.5 ng/ml TGF-\textit{β1} respectively.
| Primer sequence          | Annealing Temperature | Number of cycle | Size of PCR (bp) |
|-------------------------|-----------------------|-----------------|-----------------|
| α1(I) collagen          | 59°C                  | 32              | 177             |
| (F) 5'GGCAACAGTCGATTCACC3' |                      |                 |                 |
| (R) 5'AGGGCCAATGTCCATTCCG3' |                      |                 |                 |
| α1(III) collagen        | 60°C                  | 35              | 498             |
| (F) 5'CCTGGACCTCAGGGTATC3' |                      |                 |                 |
| (R) 5'TGCAGGGCCTGGACTACC3' |                      |                 |                 |
| α1(XIIA) collagen       | 65°C                  | 40              | 540             |
| (F) 5'AACATGCAGACCAGGCTTCCC3' |                  |                 |                 |
| (R) 5'AACCCCTCGTCTTCTCTCCCG3' |                  |                 |                 |
| α1(XIIB) collagen       | 60°C                  | 45              | 519             |
| (F) 5'AACATGCAGACCAGGCTTCCC3' |                  |                 |                 |
| (R) 5'GTATGCGGTAGTGTCACTCTGG3' |                  |                 |                 |
| α1(XII-1) collagen      | 63°C                  | 40              | 660             |
| (F) 5'GCTTTCCCAGAACATGATGCGAGCG3' |                  |                 |                 |
| (R) 5'CTCCGCAGGGGCTGGGCGAGG3' |                  |                 |                 |
| α1(XII-2) collagen      | 63°C                  | 40              | 534             |
| (F) 5'GCTTTCCCAGAACATGATGCGAGCG3' |                  |                 |                 |
| (R) 5'CAAACCTGAAGCAGCAGCTGAGAC3' |                  |                 |                 |
| TGF-β1                  | 60°C                  | 32              | 510             |
| (F) 5'GTCCGCCGCTTCTGCTCC3' |                  |                 |                 |
| (R) 5'GCCACTCAGGGCGATCAGG3' |                  |                 |                 |
| α-SM-Actin              | 60°C                  | 32              | 589             |
| (F) 5'AGGGAGTAATGTTGGAATGGG3' |                  |                 |                 |
| (R) 5'CCAGGGAGGAAGAGCGAGGCCGTGG3' |                  |                 |                 |
Table 2. Areas of fibroblasts/gel complex (% of the culture well ± SD)

|                  | No transfection control | Transfection control | Over-expressing wit3.0α | Over-expressing wit3.0β |
|------------------|-------------------------|----------------------|-------------------------|-------------------------|
| (NIH3T3)         | (NIH3T3 + BAP)          | (NIH3T3 + wit3.0α)   | (NIH3T3 + wit3.0β)      |                         |
| 6 hours          | 97.8±1.1%               | 96.7±2.7%            | 85.2±2.5%*              | 84.0±1.1%*              |
| 9 hours          | 70.2±0.9%               | 72.5±3.5%            | 63.2±1.9%*              | 59.9±3.0%*              |
| 12 hours         | 60.6±2.2%               | 61.3±1.9%            | 49.2±2.8%*              | 45.7±0.9%*              |
| 15 hours         | 45.7±0.6%               | 46.9±1.6%            | 36.9±0.1%*              | 35.6±0.8%*              |
| 18 hours         | 38.0±0.8%               | 35.6±0.6%            | 30.9±0.3%*              | 26.2±1.2%*              |
| 21 hours         | 32.9±0.9%               | 30.5±0.4%            | 27.0±1.2%*              | 23.4±1.5%*              |
| 24 hours         | 24.9±0.4%               | 23.0±1.1%            | 19.9±0.2%*              | 17.3±1.0%*              |

*P<0.05 as compared to the no transfection control
Oral fibroblast expression of wound inducible transcript-3.0 (wit3.0) accelerates the collagen gel contraction in vitro
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