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Insulin-like growth factor binding protein-5 enhances the migration and differentiation of gingival epithelial cells

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Background and Objective: The objective was to define the roles of insulin-like growth factor binding protein-5 (IGFBP-5) in gingival epithelial cells (GEC). Human IGFBP-5 is expressed in many cell types and has diverse biological functions. It stimulates the growth of bone cells and is associated with the impedance of gingival fibroblast apoptosis. In gingival epithelium, IGFBP-5 is expressed in the cells of the differentiated stratum spinosum layer.

Material and Methods: Recombinant IGFBP-5 protein treatment and knockdown of IGFBP-5 expression using a lentivirus-delivered short hairpin RNA was carried out in human GEC. Proliferation, apoptosis, anoikis, migration, differentiation and gene expression in GEC were analyzed and molecular images were obtained.

Results: The IGFBP-5 had no effect on proliferation, but it slightly suppressed apoptosis and anoikis of GEC. It also induced GEC migration and upregulated the expression of involucrin, transglutaminase-1, keratin and focal adhesion kinase. The IGFBP-5 induced migration partly via an insulin-like growth factor-independent mechanism. The knockdown of IGFBP-5 downregulated the expression of involucrin, transglutaminase-1 and focal adhesion kinase.

Conclusion: Expression of IGFBP-5 in GEC is associated with anti-apoptosis, migration and differentiation of GEC. These phenotypic effects may be associated with focal adhesion kinase and are advantageous for re-epithelization of GEC and the maintenance of gingival health.

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Insulin-like growth factors (IGFs) are a family of growth factors, receptors and binding proteins that are involved in the regulation of various physiological processes and in pathogenic conditions including the status of oral tissues (1,2). The gene for human insulin-like growth factor binding protein-5 (IGFBP-5) encodes a 30–36 kDa protein that binds to IGF and affects IGF activity (3). The IGFBP-5 may also function independently as a growth factor (4). Many human cell types express IGFBP-5, which has diverse biological functions (5,6). The variable effects might also be due to the presence of protease that degrades IGFBP-5, or via interaction with the extracellular matrix (7). The IGFBP-5 stimulates the growth of bone cells partly via an IGF-independent mechanism (8), and is also associated with the differentiation of osteoblasts and mammary epithelial cells (9–11). In gingival fibroblasts, the apoptotic cells induced by IGF-1 are significantly decreased by exogenous IGFBP-5 (12).

Expression of IGFBP-5 in periodontal ligament fibroblasts (PDLF) is higher than in gingival fibroblasts (13); however, its function in PDLF remains to be determined. Insulin-like growth factor binding protein-5 has effects in growth inhibition and migration induction of oral cancer cells. The induction of migration seemed to be independent of IGF (2).

Keratinocyte differentiation has been modeled *in vitro* (14,15). When cultured in a medium low in Ca^{2+} (< 0.1 mM), gingival epithelial cells (GEC) grow as a monolayer of polygonal cells, which can be cultivated for six or seven passages (representing approximately 20 population doublings; 16,17). In the initial passage, only a scarce amount of cytosolic involucrin is present, but it is abundantly dispersed in the cytosol, especially in the cellular cortex, during the subsequent three or four passages, during which differentiation occurs (5). Involucrin is a highly insoluble protein component of the impermeable cornified envelope (CE) that functions as a barrier (18). High levels of Ca^{2+} also upregulate another differentiation marker, transglutaminase-1 (TGM-1) (19,20), which catalyzes amide cross-linkage between glutamine and lysine residues on precursor proteins to form CE (18).

In a previous study, we demonstrated that IGFBP-5 is consistently expressed in primary human oral keratinocytes (NHOK), where it is upregulated during differentiation (5). After epithelial injury, the re-epithelization that occurs requires the coordination of the complex machineries that regulate epithelial migration and differentiation, initially to cover the wound area and then to permit cell growth to form a multilayered epithelium (21). Focal adhesion kinase (FAK) is a cytosolic tyrosine kinase localized to regions called focal adhesions (22). Tyrosine phosphorylation provides stimuli to integrins and growth factors; the subsequent activation of FAK regulates multiple biological functions (22–25). In the present study, we demonstrate that IGFBP-5 upregulates FAK expression. We also specify the roles of IGFBP-5 in promoting survival, differentiation and *in vitro* wound healing of GEC, which might be beneficial for the re-epithelization and the maintenance of gingival health.

Material and methods

Cell culture

Gingival tissue was removed from human donors who received surgery to remove an impacted third molar. The sampling protocol was approved by an Institute Review Board. Gingival epithelial cells were dissociated from the tissue and grown in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA, USA) in the presence of 0.1 mM Ca^{2+} as previously described (5). The cells were passaged when 70% confluence was reached to avoid confluence-induced differentiation (5). Cells were maintained at 37°C in an atmosphere of 5% CO_2. Gingival epithelial cell differentiation was induced by 1 mM Ca^{2+} for 24 h (26). Cells were treated with various concentrations of IGFBP-5 (Upstate Biotech, Charlottesville, VA, USA) for various times. Recombinant IGF-I (Upstate Biotech) and IGF-E3R (Peprotech, London, UK), a human IGF-I analogue having IGF activity but lacking IGFBP binding affinity, were used. An equivalent amount of 10 mM acetic acid (Sigma-Aldrich, St Louis, MO, USA) was used as a vehicle control.

Bromodeoxyuridine (BrdU) incorporation assay

Incorporation of BrdU (Sigma-Aldrich) was determined as previously described (17). Fluorescent nuclei were scored as positive, and the percentage of total nuclei that were fluorescent was determined.

Apoptosis analysis

Cells were fixed in 95% ethanol and stained with 1 g/mL of bisbenzimide (Hoechst 33258; Invitrogen) to visualize apoptotic nuclei by fluorescence microscopy (27). Apoptotic cells were identified based on nuclear morphology and were quantified as the percentage of total cells. The counting was blinded, and more than 1000 cells were counted in each sample.

Anoikis analysis

Anoikis is cell death that is induced by non-attached culture conditions (28). The anoikis assay for GEC was modified from the published protocol (27). Cells were cultured in multi-well plates coated with poly(2-hydroxyethylmethacrylate) (poly-HEMA; Sigma-Aldrich); 1×10^4 cells were seeded in each well with serum-free media and IGFBP-5. Cells harvested by pipetting and centrifugation were exposed to trypsin and ethylenediamineteraacetic acid to obtain a single cell suspension. Cell viability was determined by trypan blue exclusion (16) with an improved Neubauer haemocytometer (Metertech, Taipei, Taiwan).

Transwell cell migration assay

Cells were seeded in the upper chamber of a Transwell™ apparatus (Corning, Corning, NY, USA) on a porous transparent polyethylene terephthalate membrane (8.0 μm pore size). Hydroxurea (Sigma-Aldrich) was added to the media to a final concentration of 1 μM to terminate cell proliferation. To induce GEC migration, the opposite side of each Transwell™ membrane and the bottom of the lower chamber was coated with 20 μg/mL fibronectin (Sigma-Aldrich) for 1 h at 37°C (29). Migration of cells from one side of the membrane to the other was revealed and quantified following Hoechst 33258 staining and fluorescence microscopy (29).

Scratch wound healing assay

Gingival epithelial cells were seeded on eight-well chamber slides (Nalge Nunc, Naperville, IL, USA) coated with 0.4 mg/mL rat tail collagen (BD Biosciences, Franklin Lakes, NJ, USA), and were grown to 80% confluence. Cells were treated with 1 μM hydroxyurea to terminate proliferation. Each well was scratch wounded using a 10 μL pipette tip. Following the treatment with IGFBP-5, IGF-I, IGF-E3R

and their combination, the wound spaces were marked at the start time, and photographed at 0, 5 and 7 h using a Nikon inverted microscope (Nikon, Tokyo, Japan). Cells that migrated to wound spaces were counted.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Complementary DNA equivalent to 0.2 μg of RNA was used to analyze mRNA expression of *IGFBP-5*, *involucrin* and *TGM-1*. The primer sequences and reaction conditions are summarized in Table 1. The mRNA expression of the tested genes was normalized relative to the gene for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and quantified.

Confocal scanning microscopy

Gingival epithelial cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min and permeablized with 0.5% Triton X-100 for 15 min at 25°C. Cells were incubated with anti-FAK (1:200; Santa Cruz Biotechology, Santa Cruz, CA, USA), anti-IGFBP-5 (1:40; Santa Cruz Biotechnology), anti-involucrin (1:200; NovoCastra, Newcastle, UK), anti-pan-cytokeratin (MDBio Inc., Taipei, Taiwan) and anti-TGM1 (1:100; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (Invitrogen). Nuclei were labeled with propidium iodine (Invitrogen) or Hoechst 33258. Immunofluorescence was photographed on a Leica TCS SP2 confocal laser-scanning microscope (Leica, Heidelberg, Germany).

Short hairpin GFBP-5i lentivirus and infection

The short hairpin RNA (shRNA) vector for the knockdown of IGFBP-5 (sh*IGFBP-5*; targeting sequence of IGFBP-5 exon 1/2: 5′-GCAAGTCA-AGATCGAGAGAGA-3′) was obtained from the RNA interference consortium library (Academia Sinica, Taipei, Taiwan). The sh*Luc* vector obtained from the consortium library was used as a control. Lentivirus was generated by co-transfecting 293T cells (CRL-11268, American Type Culture Collection, Manassas, VA, USA) with lentiviral vector and packaging DNA. The lentiviruses were then used to infect GEC for 24 h in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The infected cells were grown for 72 h in the presence of 1 μg/mL puromycin (Sigma-Aldrich).

Genomic DNA was purified with the QIAGEN DNeasy™ kit (Qiagen, Valencia, CA, USA) 12 h after infection for late-RT quantitative (Q-) PCR. The analysis utilized a previously described fluorescence-monitored *Taq*Man™ PCR strategy (30) to detect reverse transcription of the virus, which represents the genomic integration that occurs as a late event of viral infection (30). The primer sets were: forward, 5′-TGTGTGCCCGT-CTGTTGTGT-3′; reverse, 5′-GAG-TCCTGCGTCGAGAGAGC-3′ and probe, 5′-(FAM)-CAGTGGCGCCC-GAACAGGGA-(TAMRA)-3′. Reactions contained 1× *Taq*Man™ universal master mix (Perkin-Elmer, Franklin Lakes, NJ, USA), 300 nM forward primer, 300 nM reverse primer, 100 nM probe primer and 500 ng of template DNA in a 30 μL volume. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 15 s at 95°C followed by 1 min at 60°C. The PCR reaction was carried out in a Quantica® real time nucleic acid detection system (Techne Inc., Burlington, NJ, USA).

Table 1. Primer sequences for RT-PCR analysis

Gene	Primer sequence (5′-3′)	Annealing temp. (°C)	Product size (bp)
IGFBP-5	Sense: TACCTGCCCAATTGTGACC Antisense: AACGTTGCTGCTGTCGAAG	54	171
Involucrin	Sense: TGAATAGTGACAAGGTGTACTGGCA Antisense: GTGGCCTGAGACATTGAGCAGCAT	65	524
TGM-1	Sense: TGTTCCTCCTCCAGTCAATACCC Antisense: ATTCCTCATGCTGTTCCCAGTGC	56	226
GAPDH	Sense: TGGTATCGTGGAAGGACTCATGAC Antisense: ATGCCAGTGAGCTTCCCGTTCAGC	55	189

Western blot

Western blot analysis followed a previously used protocol (17). The primary antibodies were those against IGFBP-5 (1:50) and TGM-1 (1:200; both were from Santa Cruz Biotechnology), involucrin (1:500; NovoCastra) and GAPDH (1:10,000; Chemicon, Temecula, CA, USA).

Statistics

The variables were analyzed with the non-parametric Mann–Whitney *U* test to compare the differences between test and control groups. Analysis across various groups (marked by a horizontal line in the figures) was also performed by the same test. Differences were considered statistically significant when $p < 0.05$.

Results

IGFBP-5 has no effect on proliferation but slightly suppresses apoptosis and anoikis of GEC

Owing to the limited life span of GEC (17), a total of 13 GEC lines, designated GEC#1 to GEC#13, were established from 13 donors in this study for various analyses. There were variations in the cellular responses among different GECs to IGFBP-5 treatment. However, the trend of responses did not vary among GECs. Gingival epithelial cells treated with 10–40 nM IGFBP-5 for 24 h displayed no change in proliferation as reflected by the presence of approximately one-third of BrdU-labeled cells in each treatment (Fig. 1A). Gingival epithelial cells treated with 40 nM IGFBP-5 for 24 h showed a slight decrease in apoptosis (Fig. 1B, $p = 0.03$) and a

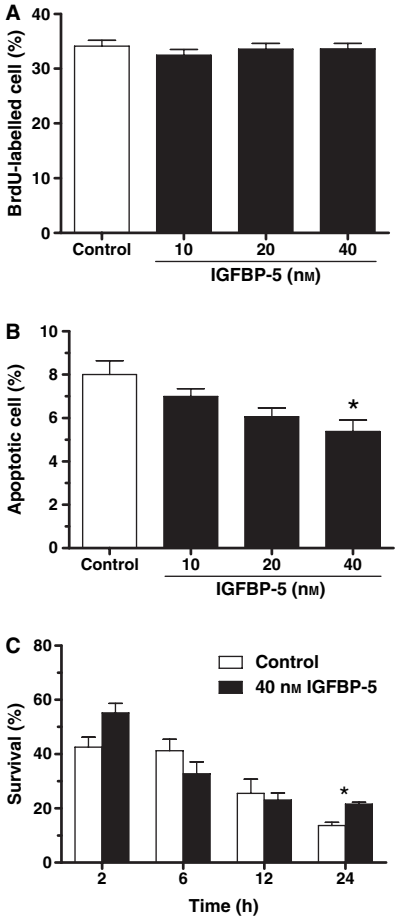

Fig. 1. Insulin-like growth factor binding protein-5 decreases GEC apoptosis and anoikis. Gingival epithelial cells were treated with 10–40 nM IGFBP-5 for up to 24 h. (A) Results of BrdU incorporation assay. The percentage of BrdU labeled cells was the same following treatment with all tested concentrations of IGFBP-5. (B) Results of apoptosis assay. Treatment with 40 nM IGFBP-5 significantly decreased the percentage of cells displaying apoptosis. (C) Results of anoikis assay. Treatment with 40 nM IGFBP-5 significantly decreased GEC anoikis. Values are expressed as means + SE from three individual GECs. $*p < 0.05$; $**p < 0.01$; Mann–Whitney *U* test.

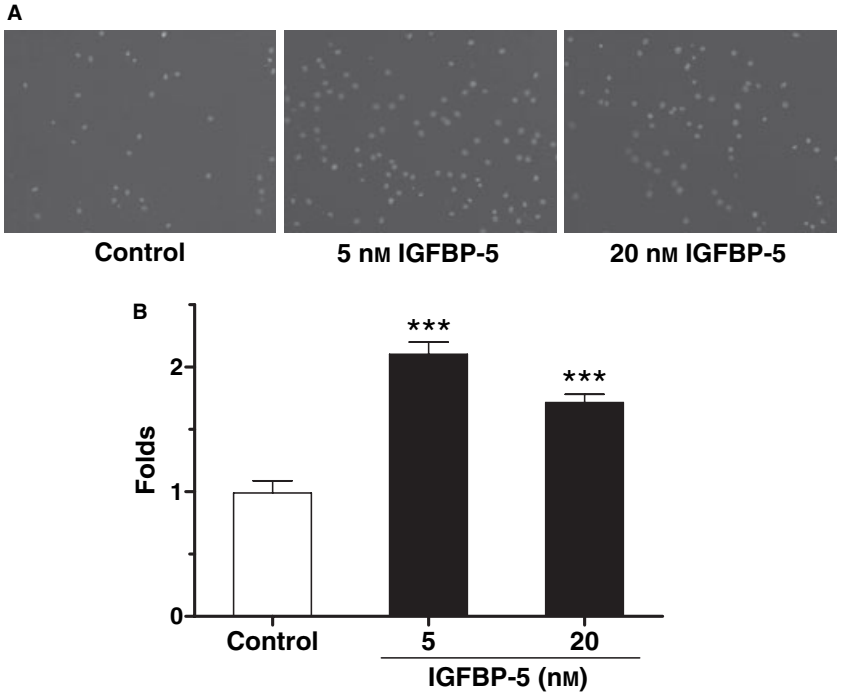

Fig. 2. Insulin-like growth factor binding protein-5 increases GEC migration. Gingival epithelial cells were treated with 5 or 20 nM IGFBP-5 for various time periods. The outer site of the Transwell™ membrane was excised, stained with Hoechst 33258, photographed, and the number of migrating cells was determined. (A) Representative fields of GEC 4 (×100). (B) Quantitation. After normalization to the control value, a significant increase of migrating GEC following IGFBP-5 treatment was evident. The values are expressed as means + SE from at least nine randomly selected fields from three individual GECs. $***p < 0.001$; Mann–Whitney *U* test.

significant increase in resistance of the cells to anoikis (Fig. 1C, $p = 0.006$).

IGFBP-5 induces GEC migration and wound healing

Cells that migrated to the outer membrane were stained with Hoechst 33258 for counting (Fig. 2A). Treatment with 5 and 20 nM IGFBP-5 for 4 h increased GEC migration in transwell migration assays (Fig. 2B; $p < 0.001$). Scratch wound healing assays were used to evaluate the migration of GEC towards the wounded area. This confirmed the marked increase in cell migration following 5 nM IGFBP-5 treatment for 5 h (not shown) and 7 h after wounding (Fig. 3). Treatment with 10 nM IGF-I or IGF-ER3 also increased GEC migration. The induction of migration by concomitant IGF-E3R and IGFBP-5 treatment on GEC for 7 h was slightly higher than with solitary IGF-E3R treatment (Fig. 3B, $p < 0.05$). However, concomitant IGF-I and IGFBP-5 treatment did not induce GEC migration. The results might suggest that both IGF-I and IGFBP-5 can induce GEC migration. Treatment with IGFBP-5 could induce IGF-I-independent migration in GEC, while the binding of IGFBP-5 to IGF-I remarkably prevented this migration, probably due to the mutual consumption of these two proteins.

IGFBP-5 upregulates involucrin, TGM-1, cytokeratin and FAK in GEC

Gingival epithelial cells were treated with various concentrations of IGFBP-5 or with 1 mM Ca^{2+} for 24 h (the latter as a positive control for differentiation). The mRNA expression of *involucrin* and *TGM-1* was investigated by RT-PCR. Following Ca^{2+} treatment, *involucrin* and *TGM-1* mRNA expression were both significantly increased (Fig. 4A,B, respectively), and the cells clustered and became flattened and squamoid. Despite the absence of apparent morphological change, 5 or 10 nM IGFBP-5 treatment for 24 h significantly increased mRNA expression of both *involucrin* and *TGM-1* mRNA ($p < 0.05$ and $p < 0.01$,

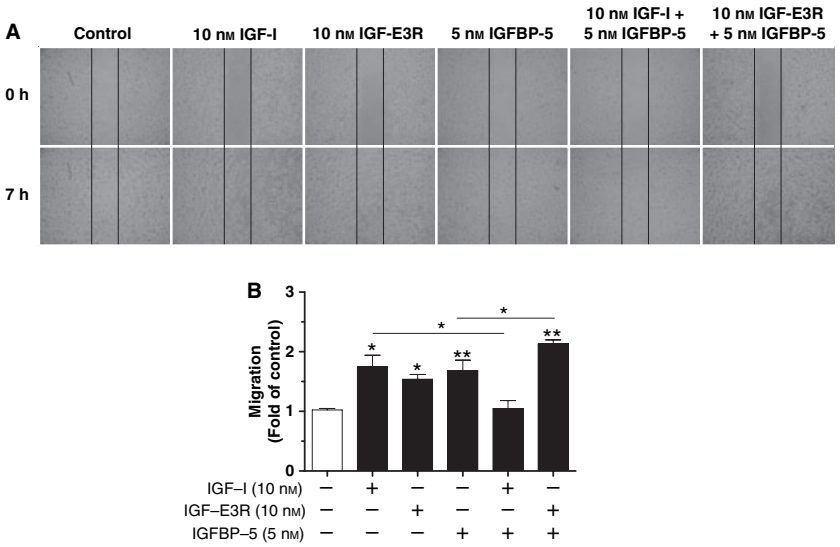

Fig. 3. Insulin-like growth factor binding protein-5 increases GEC wound healing. Gingival epithelial cells were treated with 5 nM IGFBP-5, 10 nM IGF-I, 10 nM IGF-E3R or their combination for various time periods. (A) Representative wound spaces in GEC 12 (×100). Upper panels, 0 h; lower panels, 7 h. Vertical lines designate the margins of wound spaces. The width of the wound space was measured to be 250 μm using a microruler. (B) Quantitation of GEC#12. After normalization to the control value, more rapid filling of the wound spaces in IGFBP-5-treated, IGF-I-treated or IGF-E3R-treated GEC was noted. Combined IGFBP-5 and IGF-E3R treatment further enhanced the cell migration to wound spaces. However, combined IGFBP-5 and IGF-I treatment did not induce GEC migration. The values are expressed as means + SE from at least five randomly selected wound areas. Lines indicate the test groups for comparison. $^{**}p < 0.01$; $^{***}p < 0.001$; Mann–Whitney *U* test. The study was performed in two other GECs and achieved similar results.

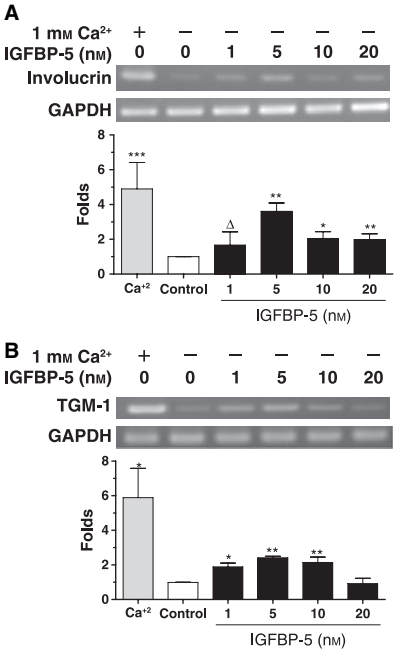

Fig. 4. Insulin-like growth factor binding protein-5 increases *involucrin* and *TGM-1* mRNA expression. Gingival epithelial cells were untreated or treated with up to 20 nM IGFBP-5 for 24 h prior to RT-PCR. Treatment with 1 mM Ca^{2+} was used as a differentiation control. *Involucrin* mRNA expression (A) and *TGM-1* mRNA expression (B) in GEC9. Upper panels show representative gel electrophoresis. After normalization to control values, upregulation of *involucrin* (lower panel of A) and *TGM-1* mRNA expression (lower panel of B) was evident following exposure to 5–20 and 1–10 nM IGFBP-5, respectively. △ Data of two GECs; statistical analysis was not performed. The other values are expressed as means + SE from at least three individual GEC. $^{*}p < 0.05$; $^{**}p < 0.01$;$^{***}p < 0.001$; Mann–Whitney *U* test.

respectively). Immunofluoresence examinations also showed that these treatments increased involucrin, TGM-1, cytokeratin and FAK protein expression in GEC (Fig. 5A–D, respectively).

Knockdown of IGFBP-5 downregulates involucrin and TGM-1 expression

To confirm whether IGFBP-5 regulates involucrin and TGM-1 expression, knockdown of endogenous IGFBP-5 in GEC using a lentiviral system was performed to monitor the subsequent effects on involucrin, TGM-1 and FAK. After infection and selection, DNAs from infected GEC were subjected to late-RT Q-PCR analysis to confirm the virus integration (not shown). Gingival epithelial cells housing integrated shRNA were designated sh*IGFBP-5*i or sh*Luc*i (control). Downregulation of *IGFBP-5* mRNA in sh*IGFBP-5*i for ~30% relative to sh*Luc*i was detected (Fig. 6A). The mRNA expression of *involucrin* and *TGM-1* was also downregulated at different levels in sh*IGFBP-5*i relative to sh*Luc*i. Western blot analysis further demonstrated the downregulation of involucrin, TGM-1 and FAK protein in a sh*IGFBP-5*i relative to sh*Luc*i (Fig. 6B). The association confirmed the causative roles of IGFBP-5 in regulating these proteins.

Discussion

Gene expression in different cell layers in stratified epithelium is believed to be associated with the phenotypes of each layer of cells. Differential IGFBP-5 expression is present in gingival epithelium, particularly in stratum spinosum, while expression is undetectable in basal or superficial cells (5). The lack of association between IGFBP-5 and GEC proliferation presently identified may be consistent with the absence of IGFBP-5 expression in basal cells of gingival epithelium. We previously observed that the differentiation of NHOK was conspicuous during the first few passages; and the cells ultimately grew to terminal differentiation during late passages (4). Interestingly, IGFBP-5 is upregulated during the differentiation stage, whereas it is diminished in terminal differentiation (5). Apoptosis has been noted in association with the terminal differentiation of oral kerainocytes (31). In the present study, IGFBP-5 suppressed apoptosis in attached and

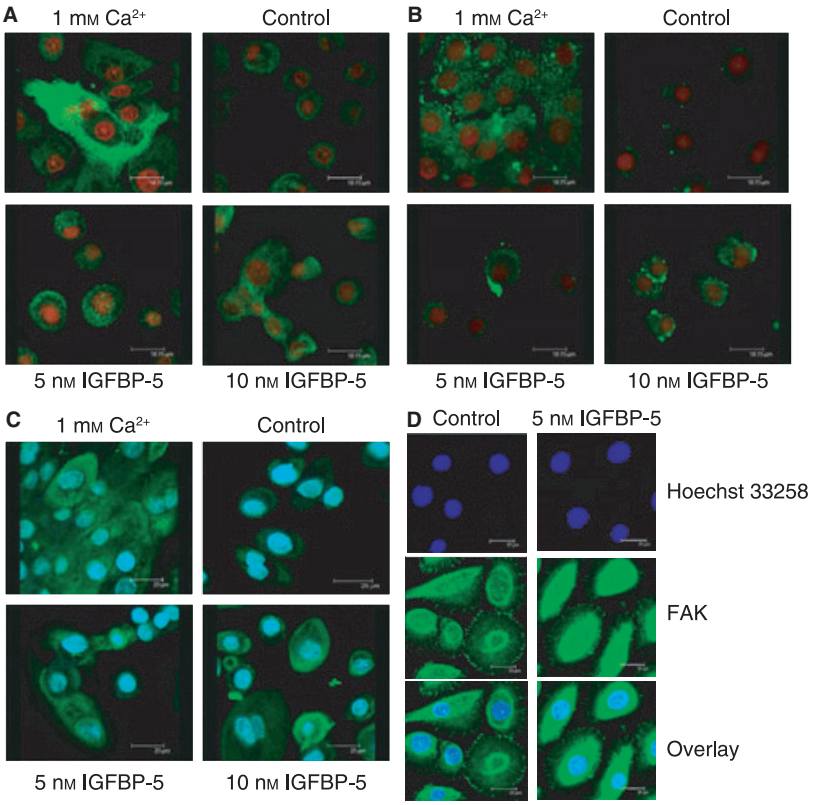

Fig. 5. Insulin-like growth factor binding protein-5 increases the immunoreactivity of involucrin, TGM-1, cytokeratin and FAK. Cell line GEC9 was untreated or treated with up to 10 nM IGFBP-5 for 24 h. Treatment with 1 mM Ca^{2+} was used as a differentiation control. (A–D) Confocal scanning microscopy for the immunoreactivity of involucrin, TGM-1, cytokeratin and FAK, respectively. Nuclei were labeled with propidium iodine (A,B) or with Hoechst 33258 (C,D). (A–C) Overlay of Alexa Fluor 488 fluorescence and nuclear image. To facilitate comparison, the immureactivity in these pictures was adjusted to a minimal level. Increase of the immunoreactivity of involucrin, TGM-1, cytokeratin and FAK was seen in GEC following IGFBP-5 treatment. Sacle bars represent 18.8 μm (A,B) or 25.0 μm (C,D). The study was also performed in GEC#13 and achieved similar results.

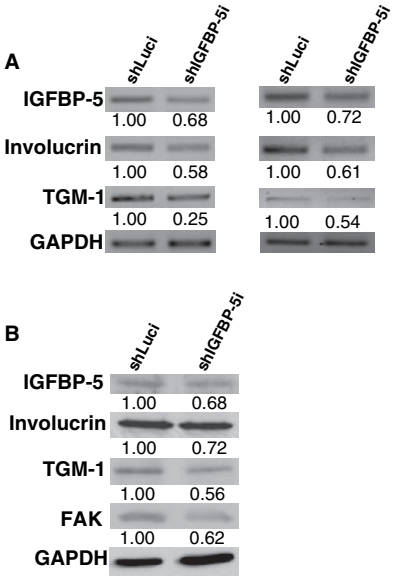

Fig. 6. Knockdown of IGFBP-5 decreases the expression of *involucrin*, *TGM-1* and *FAK*. The sh*IGFBP-5*i and sh*Luc*i were subjected to analysis. (A) RT-PCR analysis for GEC1 (left panel) and GEC#2 (right panel). The normalized mRNA expression levels are labeled below the gel pictures. (B) Western blot analysis for GEC#3. The normalized protein levels are labeled below the gel pictures. Decrease of involucrin, TGM-1 and FAK expression was seen in GECs with the knockdown of IGFBP-5.

unattached growth conditions. Since IGFBP-5 expression was remarkably attenuated during terminal differentiation, the attenuation may contribute to apoptosis occurring during terminal differentiation of GEC. Other unspecified molecules may also be involved in the regulation of terminal differentiation (31).

Our previous study has identified a link between high IGFBP-5 expression and the differentiation of NHOK and SCC25 oral cancer cells (5). However, the way in which IGFBP-5 precisely modulates differentiation of oral keratinocytes has not been defined. Treatments using exogenous recombinant IGFBP-5 protein and the administration of shRNA to curtail endogenous IGFBP-5 expression have unequivocally shown that IGFBP-5 plays critical roles in regulating GEC for the expression of involucrin and TGM-1 (32). The increase of those proteins may form cornified envelope as the barrier for the gingival mucosa (26). Although the induction of involucin, TGM-1 and cytokeratin by IGFBP-5 was not as high as calcium, and we did not notice obvious morphological changes or genesis of cornified envelope in IGFBP-5-treated GEC, the results may suggest that the induction of these differentiation phenotypes could be a later event that requires a more complicated molecular regulation in addition to IGFBP-5. Alternatively, IGFBP-5 could specifically play roles in the earlier stage of differentiation rather than in terminal differentiation. Using the same knockdown strategies and under the same selection conditions, the knockdown of IGFBP-5 in GEC was relatively limited, and was much less than the stable reduction of IGFBP-5 expression by more than 50% in oral cancer cells (2). Differences in target cells may underlie the discrepancies, or they may result from the difference in the requirement of IGFBP-5 among different types of cells for survival in selection conditions.

Insulin-like growth factor binding protein-5 can induce the differentiation of 05/50–K8 mouse osteosarcoma cells (10). A BMP-7-responsive element in the IGFBP-5 promoter is involved in regulating IGFBP-5 expression in bone cells (9). Our previous study has shown an association between calcium treatment and IGFBP-5 upregulation (5). The present study indicates that the calcium-induced differentiation of GEC may act partly through the

production of IGFBP-5. CCAAT euhacerbinding protein alpha (C/EBPα) is a key transcription factor regulating differentiation, including the differentiation of keratinocytes (33,34). Since there are C/EBPα binding elements localized immediately upstream of the TATA box in *IGFBP-5* promoter (35), calcium-associated C/EBPα activation may underlie the IGFBP-5 upregulation and the subsequent involucrin and TGM-1 expression in GEC (5). Our previous study has specified that tea polyphenol epigallocatechin-3-gallate (EGCG) can upregulate IGFBP-5 in NHOK cells (5). Since EGCG also upregulates involucrin and TGM-1 (36), it is very possible that the increase of IGFBP-5 contributes to this upregulation.

During the differentiation process of stratified squamous epithelium, keratinocytes also migrate from the basal layer to the surface (37) and display a limited number of divisions. Both transwell assay and scratch wound healing assay have shown that IGFBP-5 promotes GEC migration. The fact that the combined IGFBP-5 and IGF-E3R treatment induces more migration than the solitary IGF-E3R treatment (Fig. 3C) may further substantiate that IGFBP-5 can induce GEC migration in an IGF-independent manner. The induction of cell migration by IGFBP-5 in oral cancer cells was also mostly IGF independent (2). Berfield *et al.* (38) have shown that the IGFBP-5 carboxy-terminal peptide induces the IGF-independent migration, cell division cycle related protein 42 (cdc42) activation and cytoskeleton reorganization in mesangial cells. The activation of IGFBP-5 receptor and signal activation of GEC underlying the induction of migration requires further study. Focal adhesion kinase is a critical component in transducing signals downstream of multiple stimuli (22). Our analysis revealed an increase in FAK expression following IGFBP-5 induction. The association of FAK with oncoprotein src leads to activation of downstream signals, such as extracellular signal-regulated protein kinase (ERK), that may impact proliferation, survival, differentiation and migration of cells (24,25). Knockout of FAK in dermal keratinocytes produces marked apoptosis (23). In view of the fact that FAK impacts multiple phenotypes, the induction of survival, differentiation and migration of GES by IGFBP-5 could be associated with FAK upregulation.

Epithelial wound healing involves complicated events, including proliferation, migration and differentiation (21). Evidence from scratch wound healing, survival, migration and differentiation assays favors the view that IGFBP-5 might enhance the healing of gingival epithelium. Since IGFBP-5 is also beneficial for the proliferation, survival or differentiation of gingival fibroblasts and bone cells (8,9,12,21), it is likely that IGFBP-5 may have potential in facilitating the healing or promoting the health of periodontium.

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