Proepithelin Stimulates Growth Plate Chondrogenesis via Nuclear Factor-κB-p65-dependent Mechanisms*

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Proepithelin, a previously unrecognized growth factor in cartilage, has recently emerged as an important regulator for cartilage formation and function. In the present study, we provide several lines of evidence in proepithelin-mediated induction of chondrocytes in the growth plate lead to the formation of new cartilage (1). Simultaneously, whereas the terminally differentiated chondrocytes undergo apoptosis, the metaphysis invades the growth plate with blood vessels and bone cell precursors. The net growth plate growth is thought to be primarily on the rate of new cartilage formation (chondrogenesis) in the growth plate. The bone lengthens through a continuous, highly spatially oriented process in the growth plate of the growth plate chondrocytes and is absent from osteocytes, perichondrium, and metaphysis. The net amount of cartilage, is in part regulated by the proliferation, differentiation, and apoptosis of growth plate chondrocytes, proepithelin-mediated stimulation of metatarsal bone growth, and function. In the present study, we provide evidence to support this hypothesis that endogenously produced proepithelin by chondrocytes is important for chondrocyte growth in serum-deprived conditions. These results support the hypothesis that the induction of nuclear transcription factors (3). Proepithelin, a newly identified growth factor in cartilage, is the only growth factor able to bypass the insulin-like growth factor receptor, thus promoting growth of R- cells, which are mouse embryo fibroblasts derived from mice with targeted deletion of the insulin-like growth factor receptor gene. However, proepithelin does not protect R- cells from anchorage-independent apoptosis (anoikis) (8, 9). Conversely, in SW13 carcinoma cells, the activation of PI3K and MAPK pathways, which is proepithelin-dependent, protects cells from anoikis, confers anchorage-independent growth, and promotes tumor formation in nude mice (10, 11).

The rate of longitudinal bone growth in mammals depends primarily on the rate of new cartilage formation (chondrogenesis) in the growth plate. The bone lengthens through a continuous, highly spatially oriented process in the growth plate of the growth plate chondrocytes and is absent from osteocytes, perichondrium, and metaphysis. The net amount of cartilage, is in part regulated by the proliferation, differentiation, and apoptosis of growth plate chondrocytes, proepithelin-mediated stimulation of metatarsal bone growth, and function. In the present study, we provide evidence to support this hypothesis that endogenously produced proepithelin by chondrocytes is important for chondrocyte growth in serum-deprived conditions. These results support the hypothesis that the induction of nuclear transcription factors (3).

This article has been withdrawn by the authors. The authors were recently made aware that in Fig. 6B, the cytoplasmic p65 lane at 12 h was reused as the nuclear Lamin B lane at 12 h, flipped horizontally. The cytoplasmic Lamin B lane at 12 h was reused as the nuclear GAPDH lane at 0 h. In Fig. 7A, the third lane in the Proepithelin immunoblot appeared to be spliced. The authors affirm that all experiments were performed appropriately, and they state that experimental data generated in the laboratory from the same period support the original conclusions of the study. The authors state that these issues do not affect the accuracy of the overall results or the conclusions of the scientific work. However, in the interest of maintaining the accuracy in the published scientific literature, the authors wish to withdraw this article and will seek to publish a new version of the article corroborating the findings of this work in the future.

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The activation of Akt is IRS-1-independent, suggesting that proepithelin activates AKT through an unidentified pathway (12, 13). We recently showed that the NF-κB subunit p65 facilitates growth plate chondrogenesis via the PI3K/Akt pathway (14). In addition, NF-κB exerts a regulatory role in bone growth and development in a way different from that of the tyrosine-kinase receptor (15). Mice deficient in NF-κB subunits p50 and p52 show retarded growth and shortened long bones (16). We hypothesize that proepithelin plays a critical role in chondrocyte development via an unidentified pathway such as NF-κB.

We show that proepithelin and pyrrolidine dithiocarbamate (PDTC, a known NF-κB inhibitor) affect metatarsal longitudinal growth and growth plate chondrogenesis. Furthermore, we show the effects of proepithelin on the nuclear translocation of NF-κB in growth plate chondrocytes and the effects of depletion of endogenous proepithelin and NF-κB-p65 on cultured growth plate chondrocyte proliferation, differentiation, and apoptosis. In addition, we evaluate the potential intracellular signaling pathways required for the proepithelin-mediated induction of NF-κB activity. Our results support the hypothesis that the induction of NF-κB activity in growth plate chondrocytes is critical in proepithelin-mediated growth plate chondrogenesis and longitudinal bone growth.

**EXPERIMENTAL PROCEDURES**

**Whole Metatarsal Culture**—The second, third, and fourth metatarsal bone rudiments were isolated from Sprague-Dawley rat fetuses at 20 days post conception and cultured in 24-well plates (17, 18). Each well contained minimum essential medium (Invitrogen) supplemented with 0.05 mg/ml ascorbic acid (Sigma-Aldrich), 1 mM sodium glycero phosphate (Sigma), 0.2% bovine serum albumin (Sigma), 100 units/ml penicillin, and 10% fetal bovine serum. The bone rudiments were cultured in the absence or presence of 240 nM Enzo Life Sciences recombinant proepithelin and/or 1 μM PDTC (Sigma), a specific NF-κB inhibitor. Animal care was in compliance with the guidelines of the Institutional Animal Ethics Committee for the Care and Use of Laboratory Animals.

**Measurement of Metatarsal Longitudinal Growth**—The length of each bone rudiment was measured using a dissecting microscope with an eyepiece micrometer that was calibrated daily with a 1-mm stage micrometer. To calculate the metatarsal growth rate, bone length was measured at the beginning and at the end of the 3-day culture period using an eyepiece micrometer in a dissecting microscope. For each treatment group, 48 metatarsal bones isolated from 8 rat fetuses were used. Results represent the mean ± S.E. of three separate experiments.

**Quantitative Histological Analysis**—At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde overnight. After routine processing, three longitudinal sections, 5–7 μm thick, were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, we measured the height of the epiphyseal zone, the proliferative zone, and the hypertrophic zone and calculated the average value. In the metatarsal growth plate, the epiphyseal zone was characterized by small, rounded cells irregularly arranged in the cartilage matrix. The proliferative zone included cells with a flattened shape, arranged in columns parallel to the longitudinal axis of the bone. In the hypertrophic zone, large cells (defined by a height of 9 μm) formed a layer adjacent to the calcified region of the metatarsal bone, the primary ossification center. Results represent the mean ± S.E. of three separate experiments. Quantitative histological analysis was performed by a single observer blinded to the treatment category.

**BrdU in Situ Incorporation**—After 3 days in culture, 5-bromo-2′-deoxyuridine (BrdU) was added to the culture medium at a final concentration of 10 μM (2). Bone rudiments were incubated for an additional 5 h. At the end of the incubation, all bones were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5- to 7-μm-thick longitudinal sections. Bone sections were stained for BrdU according to the manufacturer’s protocol (Invitrogen). The BrdU-labeled number of cells per grid was determined for each growth plate zone (epiphyseal, proliferative, or hypertrophic). The number of cells in three distinct grid locations per bone was averaged. For each treatment group, 10 bones were sampled, and 3 growth plate sections of each bone were assessed. The labeling index (number of labeled cells/total cells) was determined separately for the epiphyseal zone and for the proliferative zone. All determinations were made by the same observer blinded to the treatment category.

**Chondrocyte Culture**—Metatarsal rudiments were isolated from Sprague-Dawley rat fetuses at 20 days post conception, rinsed in PBS, and incubated in 0.2% trypsin for 1 h and then in 0.2% collagenase for 3 h. The cell suspension was aspirated repeatedly, filtered through a 70-μm-thick filter, and collected in 100-mm dishes. Cells were seeded in 100-mm dishes at a density of 5 × 10^5/cm^2 in DMEM with 100 units/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10% fetal bovine serum. The cell culture medium was changed at 72-h intervals. Once 70–80% confluence was reached, cells were washed with serum-free medium and treated with purified recombinant proepithelin (240 nM) and/or 1 μM PDTC and/or selective protein kinase inhibitors (EMD Chemicals, Gibbstown, NJ) (14, 19).

**[^3H]Thymidine Incorporation**—To assess proliferation in cultured chondrocytes, 2.5 μCi/well[^3H] thymidine (Amersham Biosciences) was added to the culture medium for an additional 3 h at the end of the culture period. Cells were then washed, precipitated with trichloroacetic acid, and lysed in 0.5 M NaOH, 0.5% SDS. Incorporation of[^3H] thymidine was measured by liquid scintillation counting and normalized by protein content.

**Real-time PCR**—At the end of the culture period, total RNA was extracted from the growth plates of whole rat metatarsal

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[^3]: The abbreviations used are: PDTC, pyrrolidine dithiocarbamate; SNP, sodium nitroprusside.
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bones or from cultured chondrocytes using the RNeasy mini kit (Qiagen Inc., Valencia, CA). The following specific primers were used: rat collagen X (A131848); (forward) 5'-TCT GTA CAA CAG GCA GCA CTA-3', (reverse) 5'-GTA CAT TGT GGG GTT GCC ATT CT-3'; rat ALP (NM_013059): (forward) 5'-AAT CGG AAC AAG CTG ACT GAC CCT-3', (reverse) 5'-ATT CCT GCC TCC TTC CAC TAG CAA-3'; rat Runx2 (NM_053470): (forward) 5'-ATG ATC AGA CTG CCA CCT CTG ACT-3', (reverse) 5'-TGA GGG AAA TCG AAG CAT TTG CGG TGC ACG ATG-3'.

One microgram of total RNA and 1.6 μg of p(dT)15 primer were incubated for 10 min at 25 °C, followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. Real-time quantitative PCR was carried out using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) at a final volume of 25 μl containing 1 μl of cDNA, 12.5 μl of 2 × SYBR Green master mix (Applied Biosystems), 0.1 μM primer (Applied Biosystems) in DNase-free water. The PCR conditions were: 50 °C for 2 min and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Product sizes were determined by 40% acrylamide gel and then air-dried. Apoptotic cells were identified by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling, according to the manufacturer's instructions (TdT-FragEL kit, Oncogene Research Products, Boston, MA). A positive control was generated by adding 1 μg/μl DNase I in 1× TBS, 1 mM MgSO4 following treatment with proteinase K, whereas a negative control was generated by substituting distilled H2O for the terminal deoxynucleotidyl transferase in the reaction mixture. All other steps were performed as described above (data not shown).

In situ cell death was measured by determining the apoptotic index. The apoptotic index was calculated as the number of apoptotic cells per grid divided by the total number of cells per grid. The grid circumscribed cultured chondrocytes analyzed through a 40× objective and generally contained an average of 30 cells. For each treatment group, the apoptotic index was calculated in five distinct grid locations. Results are expressed as the mean ± S.E. of three separate experiments. Indices were calculated by a single observer blinded to the treatment regimen.

Caspase-3 Assay—Caspase-3 activity was determined in cell lysate using Tris-HCl buffer (pH 7.0), 0.5 mg/ml of caspase substrate containing the recognition sequence for caspase-3 (Ac-DEVD-AMC, Upstate Biotechnology, Inc., Lake Placid, NY). The caspase-3 substrate was cleaved fluorometrically at 460 nm and measured by fluorometry at 37 °C for 150 s. The caspase-3 activity was expressed as nanomolar protein/h.

NF-κB DNA-binding Activity—NF-κB-p65 DNA-binding activity was determined by using an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor MI, catalogue number 10007889), according to the manufacturer's instructions. A specific double-stranded DNA sequence containing the NF-κB response element was immobilized onto the well bottoms of a 96-well plate. NF-κB-p65 contained in the nuclear extract was detected by addition of a specific primary antibody directed against NF-κB-p65. A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm. Nuclei were extracted from chondrocytes treated for 24 h with purified recombinant proepithelin (240 nM), and/or 1 μM PDTC, and/or the following specific protein kinase inhibitors: wortmannin (PI3K inhibitor); U0126 (MAPK inhibitor); Akti 1/2 (Akt inhibitor); bisindolylmaleimide I (PKC inhibitor); or H-89 (PKA inhibitor). All of these inhibitors were purchased from EMD Chemicals. Data are expressed as the mean ± S.E. of the optical density per microgram of protein and represent three separate experiments.

Western Blot—Whole cell lysates were solubilized with 1% SDS sample buffer and electrophoresed on a 4–15% SDS-PAGE gel (Bio-Rad, Richmond, CA). Proteins were transferred onto a nitrocellulose membrane and probed with the following primary antibodies: rabbit polyclonal antibodies against p-STAT5 (Cell Signaling Technology Inc., Danvers, MA), goat polyclonal antibody against STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Erk1/2 (T202/Y204, Cell Signaling), anti-Erk1/2 (Cell Signaling), NF-κB-p65 (Santa Cruz}

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Biotechnology), Lamin B (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), and rabbit polyclonal antibody against \( /H9252\)-actin (Sigma-Aldrich). The blots were developed using a horseradish peroxidase-conjugated polyclonal goat-anti-rabbit IgG antibody and enhanced chemiluminescence system (Amersham Biosciences). The protein size was confirmed by molecular weight standards (Invitrogen).

**Immunofluorescence/Confocal Microscopy**—Primary cultured chondrocytes were treated with or without proepithelin for the indicated times and seeded onto glass coverslips that had been pretreated with 0.01% polyornithine (Sigma-Aldrich). Coverslips were washed twice with PBS plus Ca\(^{2+}\)/Mg\(^{2+}\). Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS plus Ca\(^{2+}\)/Mg\(^{2+}\) for 10 min. After incubation in blocking buffer (5% BSA in PBS plus Ca\(^{2+}\)/Mg\(^{2+}\)), the permeabilized chondrocytes were incubated with anti-NF-\(b\)-p65 antibody (1:200) and subsequently stained with FITC-and rhodamine-conjugated secondary antibodies (sc-2078 and sc-2095, Santa Cruz Biotechnology) for 1 h at room temperature. Cell nuclei were stained with FITC (green fluorescence). Finally, coverslips were washed with PBS three times and mounted on glass slides with Vectashield mounting medium (H-1000, Vector Laboratories Inc.). Fluorescent images were collected on a Zeiss Axiovert 100 confocal microscope using a Zeiss 40\times objective.

**ChIP Assay**—Chromatin immunoprecipitation (ChIP) assays were carried out by following a previous publication (21). Sub-confluent chondrocytes were made quiescent and then stimulated with proepithelin for up to 24 h. Following treatment, the pre-cleared chromatin was immunoprecipitated for 12 h with specific antibodies against NF-\(b\) or UBF (served as positive control). The primers used were as follows: Cyclin D1 forward, 5'-CGG ACT AAG GGG AGT TTT GTT G-3'; reverse, 5'-TCC AGC ATC AGG TGG CAC GAT-3'. For the GAPDH promoter, the primers were forward P1 (5'-AGT GCC AGC CTC GTC CCG TAG ACA AAA TG-3') and promoter reverse P2 (5'-AAG TGG GCC CCCG GCC TCC AT-3'). The amplification products were analyzed in a 2% agarose gel and visual-
RESULTS

Expression of Proepithelin in the Metatarsal Growth Plate and the Effects of Proepithelin and PDTC on Metatarsal Longitudinal Growth and Growth Plate Chondrogenesis—Because proepithelin is known as a growth factor that promotes cell-cycle progression and growth of many cellular systems (19–21), we sought to determine whether proepithelin plays a critical role as an autocrine growth factor in the establishment and progression of growth plate chondrogenesis. Therefore, we tested the expression of proepithelin in serum-free, whole metatarsal culture, because metatarsals keep growing for up to 1 week in the absence of serum, which enables us to assess the expression of proepithelin in a manner similar to that of the autocrine function.

Immunohistochemical analysis of sections of 48-h fetal rat metatarsals using the specific antibody against proepithelin showed that the whole growth plate, including epiphyseal, proliferative, and hypertrophic chondrocyte (Fig. 1, A and B), stains intensely for proepithelin. In a preliminary experiment, increasing the concentration of proepithelin from 60 to 120, 240, and 480 nM induced dose-dependent metatarsal longi-

FIGURE 2. Effect of proepithelin on cell proliferation and the expression of collagen X, ALP, and Runx2 in the metatarsal growth plate. A, after 3 days in culture, BrdU was added to the culture medium at a final concentration of 10 μM. Bone rudiments were incubated for an additional 5 h. At the end of the incubation, all bones were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5- to 7-μm-thick longitudinal sections. Bone sections were stained for BrdU according to the manufacturer's protocol. A representative BrdU-labeled cell is indicated. B, the labeling index was calculated as described under “Experimental Procedures.” For each growth plate zone, the fraction of labeled cells in three distinct grid locations was calculated and averaged. The labeling index for the epiphyseal zone and for the proliferative zone (n = 12 bones/group). C, mRNA expression of collagen X, ALP, and Runx2 mRNA were normalized by -actin in the same samples. Results are expressed as -fold change compared with untreated control metatarsal bones. Significant differences from control are indicated (mean ± S.E.).
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FIGURE 3. Effect of proepithelin on chondrocyte proliferation and collagen X expression in cultured growth plate chondrocytes. Chondrocytes were transfected with siRNA targeted for NF-κB-p65 and cultured in DMEM containing 10% FCS for 24 h, and seeded in a 24-well plate to incubate in the absence or presence of proepithelin and PDTC 1 mM. The chondrocytes were added with 0.5 μCi/well of [3H]thymidine and collected onto glass fiber filters. Incorporation of [3H]thymidine was measured by liquid scintillation counting. Results were expressed as percent incorporation compared with control siRNA. RNA was extracted from chondrocytes by real-time PCR. Total RNA was extracted from chondrocytes isolated from metatarsal bones, and reverse-transcribed to cDNA. The relative expression levels of collagen X mRNA were normalized by β-actin in the same samples. Results are expressed as the mean ± S.E. (n = 3).

Effects of Proepithelin, PDTC, and p65 siRNA on Chondrocyte Proliferation, Differentiation, and Apoptosis—To determine whether proepithelin interacts functionally with NF-κB-p65 in chondrocytes, we first transfected chondrocytes isolated from fetal rat metatarsal growth plates with rat NF-κB-p65 siRNA or control siRNA. p65 siRNA-transfected chondrocytes exhibited reduced p65 mRNA and protein expression (Fig. 3, A and B) compared with control siRNA-transfected chondrocytes.

To confirm the findings observed in the whole metatarsal bones, we evaluated the effects of proepithelin on the proliferation, differentiation, and apoptosis of the transfected growth plate chondrocytes. In chondrocytes isolated from metatarsal growth plates and transfected with control siRNA, proepithelin induced chondrocyte proliferation (assessed by [3H]thymidine incorporation; proepithelin versus control, Fig. 3C) as well as differentiation (assessed by collagen X mRNA expression, Fig. 3D). The co-treatment of proepithelin with 1 μM PDTC or the transfection with NF-κB-p65 siRNA reversed these stimulatory effects (proepithelin + PDTC versus proepithelin, Fig. 3, C and D).

In light of the regulatory role of proepithelin and NF-κB on apoptosis in other cell types, we evaluated the effects of proepithelin, PDTC, and p65 siRNA on chondrocyte apoptosis by
assessing in situ cell death and caspase-3 activity. Chondrocytes cultured with 1 mM SNP exhibited increased cell death (representative sections, Fig. 4A; apoptosis index, Fig. 4B) and caspase-3 activity (Fig. 4C) compared with control chondrocytes. The addition of proepithelin to the culture medium of the SNP-treated chondrocytes prevented SNP-mediated increase of cell death (representative sections, Fig. 4A; apoptosis index, Fig. 4B) and caspase-3 activity (Fig. 4C). However, co-treatment with 1 μM PDTC reversed the anti-apoptotic effects of proepithelin on SNP-induced cell death and caspase-3 activity.

**Effects of Proepithelin on NF-κB-p65-DNA-binding Activity and Cyclin D1 Activation**—To determine whether proepithelin specifically induces NF-κB-p65 activation, we evaluated the NF-κB-p65 transcription factor enzyme-linked immunosorbent assay. Proepithelin significantly increased the NF-κB-p65-DNA-binding activity in control siRNA-transfected cells, whereas co-treatment of chondrocyte with 1 μM PDTC and proepithelin reversed such stimulatory effect of proepithelin (Fig. 5A). Although the addition of proepithelin to the culture medium of chondrocytes previously transfected with NF-κB-p65 siRNA did not modify the NF-κB-p65 DNA-binding activity, compared with untreated chondrocytes transfected with a control siRNA (Fig. 5A).

On the basis of the preceding results, we investigated the effect of proepithelin-mediated intracellular signaling on NF-κB-p65 DNA-binding activity. We cultured chondrocytes in the presence of proepithelin, with or without specific inhibitors of each of the signaling pathways activated by proepithelin. Preliminary tests showed that the same concentration of these specific inhibitors had no toxic effect on chondrocytes growing in serum and that the concentrations used for these experiments were effectively abolishing the activation of each pathway (data not shown).

The addition of 10 μM wortmannin (a PI3K inhibitor) or 10 μM Akti1/2 (an Akt inhibitor) to the culture medium of proepithelin-treated chondrocytes significantly reversed the stimula-
tory effects of proepithelin on NF-κB DNA-binding activity (Fig. 5B). In contrast, the addition of U0126 (a MAPK inhibitor), H-89 (a PKA inhibitor), or bisindolylmaleimide I (a PKC inhibitor) did not affect the proepithelin-mediated increase of NF-κB-p65 activity (Fig. 5C). In the absence of proepithelin in the culture medium, none of these inhibitors had any effect on NF-κB-p65 DNA-binding activity in chondrocytes (Fig. 5, B and C). To further confirm which signaling pathway is involved in NF-κB activation, we detected phosphorylation of Akt and Erk upon proepithelin stimulation. Our result showed a rapid and time-dependent activation of Akt induced by proepithelin in the first 2 h of treatment, in contrast a trace level of activation of Erk1/2 signal was observed at 30 and 60 min of treatment (Fig. 6A).

To explore the relationship between NF-κB activation and chondrocytes function induced by proepithelin, we first determined NF-κB-p65 translocation upon stimulation of proepithelin. Our result clearly indicated that NF-κB-p65 translocated into nuclear upon stimulation of proepithelin, as assessed by confocal microscopy and Western blot as well (Fig. 6, B and C). Then, we determined whether NF-κB is associated with the regulatory sequences of cyclin D1 promoter by standard ChIP procedures based on the fact that cyclin D1 is known as a NF-κB induced gene that encodes molecules involved in cell proliferation. Because UBF is present in the cyclin D1 promoters, it served therefore as the positive control (21). Mouse IgG served as the negative control. The band shown in Fig. 6D is of the correct size for the selected fragment of the cyclin D1 promoter. In chondrocytes, NF-κB was detectable in cyclin D1 promoter at 12 and 24 h after proepithelin stimulation. Lastly, we performed a luciferase assay, and the result showed that proepithelin significantly induced cyclin D1 promoter luciferase activity, whereas such induction by proepithelin was nullified by the addition of NF-κB inhibitor, PDTC, further confirming that proepithelin induced cyclin D1 via NF-κB (Fig. 6E).

Proepithelin Facilitates Growth Plate Chondrogenesis—Chondrocytes that in vitro express high levels of proepithelin proliferate in the absence of serum. We performed experiments to determine whether endogenously produced proepithelin contributes to the proliferation of chondrocytes. We initially determined the

FIGURE 5. Effects of proepithelin on NF-κB DNA-binding activity. Chondrocytes were cultured in the presence or absence of proepithelin with or without specific signaling pathway inhibitors. NF-κB-p65 transcription factor activity was determined by an enzyme-linked immunosorbent assay according to the manufacturer's instructions. Results are expressed as A450/mL of nuclear protein and represent mean values obtained from three independent experiments. A, PDTC and p65 siRNA (NF-κB inhibitors); B, wortmannin (PI3K inhibitor) and Akti1/2 (Akt inhibitor); C, U0126 (MAPK inhibitor), H89 (PKA inhibitor), and bisindolylmaleimide I (BIS) (PKC inhibitor).
effect of transient proepithelin depletion on cell proliferation of chondrocytes in the absence of serum. We achieved almost complete depletion of endogenous proepithelin by transiently transfecting chondrocytes with specific siRNA for proepithelin compared with control oligonucleotides and vehicle-transfected chondrocytes (Fig. 7A). Proepithelin depletion induced a considerable reduction of chondrocyte proliferation in a serum-deprived condition of chondrocytes compared with control chondrocytes (Fig. 7B), suggesting that endogenously produced proepithelin contributes to the ability of chondrocytes to grow in the absence of serum.

DISCUSSION

Understanding the mechanisms maintaining the postnatal growth plate and articular cartilage is of both scientific and clinical significance. Our study demonstrates that proepithelin stimulates metatarsal longitudinal growth. This effect results from the stimulation of the two main cellular events of growth plate chondrocyte proliferation (reflected by the increased percentage of proliferating cells in the epiphyseal and proliferative zones, and the increased height of both zones), and chondrocyte differentiation/hypertrophy (increased hypertrophic zone height and induced collagen X mRNA expression) (22). The effects observed in the metatarsal growth plate were confirmed by experiments in chondrocytes cultured in the presence of proepithelin. The stimulatory effects of proepithelin on longitudinal bone growth may also be related to its anti-apoptotic effects in the growth plate, as suggested by the proepithelin-mediated prevention of chondrocyte apoptosis induced by sodium nitroprusside (23, 24).

Proepithelin is heavily glycosylated and appears as a ~90-kDa protein on SDS-PAGE (25). Recent evidence indicated that
Proepithelin Facilitates Growth Plate Chondrogenesis

**A**

![Image](image_url)

**B**

![Graph](graph_url)

*Proepithelin stimulates chondrocyte proliferation and inhibits cell death.*

Proepithelin mRNAs are highly expressed in growth plate cartilage, particularly in growth plate chondrocytes. Proepithelin expression is restricted to chondrocytes in the lower proliferative and upper hypertrophic zones (4). Proepithelin stimulates the proliferation of immature cartilage and cartilage oligomeric matrix protein appears to be required for proepithelin-mediated chondrocyte proliferation in cartilage (6). In vivo genetic knockdown of proepithelin led to a sharp decrease in growth plate length, chondrocyte proliferation rate, and Col2a1 and Col10a1 expression (6). More significantly, proepithelin may slow or block the degenerative events that occur in patients with arthritis by inhibiting two important molecules (ADAMTS-7 and ADAMTS-12) associated with the degradation of cartilage (7).

Because the proepithelin membrane receptor has not yet been identified, it is not possible to clearly define the early stages of proepithelin-mediated signaling from the plasma membrane. Our study demonstrated that the activation of PI3K/Akt signaling but not of the MAPK pathway is required for proepithelin-modulated chondrocyte proliferation, differentiation, and apoptosis. Our data enhance understanding of the one or more mechanisms by which PI3K/Akt contributes to the regulation of proepithelin-induced chondrogenesis. The difference in Erk1/2 pathway activation between the current study and that of Feng et al. (4) might be due to the different cell lines used. Human C28I2 chondrocyte was derived from human juvenile costal cartilage and generated by infection with a replication retroviral vector expressing SV40 large T antigen (26). It has been demonstrated that SV40 large T antigen (SV40LT) can alter some cytoplasmic signaling pathways, such as the MAPK pathway (27). Previous studies in other cell types have shown that proepithelin promotes the activation of the MAPK pathways in MCF-7 breast cancer cells (21), whereas proepithelin promotes the activation of the PI3K and MAPK pathways in mouse embryo fibroblasts, adrenal carcinoma, and multiple myeloma cells (10). Although this experimental evidence has shed light on the signaling pathways involved, the one or more transcription factors ultimately mediating the effects of proepithelin on growth plate chondrogenesis remain to be identified.

Experimental results from a number of cell types suggest interaction between PI3K/Akt and NF-κB (1, 28, 29), we hypothesized that proepithelin regulates growth plate chondrogenesis and longitudinal bone growth by inducing the activity of NF-κB in growth plate chondrocytes. We recently demonstrated that NF-κB is expressed in growth plate chondrocytes, facilitates endochondral bone growth by inducing chondrogenic and differentiation and by preventing apoptosis (5). Our findings indicate that proepithelin enhances NF-κB activation in growth plate chondrocytes. The neutralization of NF-κB activity by PDTC (a specific NF-κB inhibitor) neutralized the stimulation of growth plate longitudinal bone growth. In effect, PDTC increased the expression of basal (unstimulated) NF-κB in proepithelin-modulated chondrocytes. More importantly, the inhibition of NF-κB by PDTC alone did not affect the cellular process. Such a difference would suggest stronger inhibitory effects of proepithelin on growth plate differentiation/hypertrophy, as assessed by collagen X mRNA expression in the metatarsal growth plate and by histological analysis of the growth plate.

Our experiments in cultured chondrocytes confirmed the effects of PDTC on the proepithelin-mediated induction of cell proliferation and differentiation in the metatarsal growth plate (30, 31). Furthermore, the addition of PDTC to the culture medium reversed the anti-apoptotic effects of proepithelin in cultured chondrocytes, as assessed by in situ cell death and caspase-3 activity. Although the selective inhibition of NF-κB-p65 expression in chondrocytes by siRNA led to decreased chondrocyte proliferation, PDTC alone did not affect this cellular process. Such a difference would suggest stronger inhibition of basal (unstimulated) NF-κB-p65 activity caused by p65 siRNA compared with PDTC. The neutralization of the p65 siRNA effects on chondrocyte proliferation by proepithelin further supports a functional interaction between NF-κB-p65 and proepithelin in the regulation of growth plate chondrogenesis and longitudinal bone growth. In a chondrocytic cell line, the activation of NF-κB by PI3K/Akt was triggered by bone morphogenetic protein-2 (32, 33), another growth factor expressed in the growth plate known to stimulate growth plate chondrogenesis (34). In the current study, proepithelin stimulated nuclear translocation of NF-κB-p65 and initiated a cascade of target genes involved in cell proliferation and anti-apoptosis, which clearly demonstrated the relationship between NF-κB activation and chondrocyte function.

Previous evidence indicated that the activation of NF-κB-p65 by the PI3K/Akt pathway involves p65 Ser-536 phos-
phorylation by IκB kinase (35–37). Depletion of endogenous proepithelin had a dramatic effect in inhibiting growth of chondrocytes under conditions of serum deprivation, a condition that is close to the cellular autocrine/paracrine environment in vivo. It is therefore reasonable to speculate that proepithelin functions effectively in an autocrine manner (38), suggesting that proepithelin may be an attractive medication with little toxicity to normal cells. Further studies are required to determine the relative roles of the upstream activators and downstream effectors for NF-κB during proepithelin-mediated growth plate chondrogenesis.

In conclusion, our study suggests that proepithelin promotes longitudinal bone growth and growth plate chondrogenesis, which are mediated through NF-κB via the PI3K/Akt signaling pathway.

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