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Mutation of Spry2 Induces Proliferation and Differentiation of Osteoblasts but Inhibits Proliferation of Gingival Epithelial Cells

Terukazu Sanui,[1] Urara Tanaka,[1] Takao Fukuda,[1]* Kyosuke Toyoda,[1] Takaharu Taketomi,[2] Ryo Atomura,[1] Kensuke Yamamichi,[1] and Fusanori Nishimura[1]

[1]*Department of Periodontology, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Fukuoka, Japan*

[2]*Dental and Oral Medical Center, Kurume University School of Medicine, Kurume, Fukuoka, Japan*

ABSTRACT

Sprouty was identified as an inhibitor of the fibroblast growth factor (FGF) receptor, and Sprouty2 (Spry2) functions as a negative regulator of receptor tyrosine kinase signaling. In this study, we investigated how inhibition of Spry2 affects osteoblasts and gingival epithelial cells in periodontal tissue regeneration in vitro. Transduction of a dominant-negative mutant of Spry2 (Y55A-Spry2) enhanced basic fibroblast growth factor (bFGF)- and epidermal growth factor (EGF)-induced ERK activation in MC3T3-E1 osteoblastic cells. In contrast, it decreased their activation in GE1 cells. Consistent with these observations, Y55A-Spry2 increased osteoblast proliferation with bFGF and EGF stimulation, whereas the proliferation of Y55A-Spry2-introduced GE1 cells was decreased via the ubiquitination and degradation of EGF receptors (EGFRs). In addition, Y55A-Spry2 caused upregulation of Runx2 expression and downregulation of Twist, a negative regulator of Runx2, with treatment of bFGF and EGF, resulting in enhanced osteoblastogenesis accompanied by alkaline phosphatase activation and osteocalcin expression in MC3T3-E1 cells. These data suggest that suppression of Spry2 expression induces proliferation and differentiation of osteoblastic cells after the addition of a bFGF and EGF cocktail but inhibits proliferation in gingival epithelial cells. These in vitro experiments may provide a molecular basis for novel therapeutic approaches in periodontal tissue regeneration. Taken together, our study proposes that combined application of an inhibitor for tyrosine 55 of Spry2, bFGF, and EGF may effectively allow alveolar bone growth and block the ingrowth of gingival epithelial cells toward bony defects, biologically mimicking a barrier effect in guided tissue regeneration, with in vivo investigation in the future. J. Cell. Biochem. 116: 628–639, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: Spry2; PERIODONTAL REGENERATION; OSTEOBLASTS; GINGIVAL EPITHELIAL CELLS; bFGF; EGF

Periodontal disease is known as a chronic inflammation caused by persistent bacterial insult and it results in the destruction of gingival tissue and the absorption of alveolar bone in adults [Brown and Löe, 1993; Pihlstrom et al., 2005]. If left untreated, the disease leads to bone destruction, inducing tooth mobility and subsequent tooth loss. Many recent experimental and clinical studies of techniques for periodontal tissue regeneration have been conducted, such as guided tissue regeneration (GTR) and enamel matrix derivative (EMD) (Straumann® Emdogain). GTR is a surgical procedure using a barrier membrane that is placed over the bony defect to prevent gingival epithelial downgrowth into the wound space, thereby maintaining a space for periodontal tissue regeneration [Ratka-Krüger et al., 2000; Villar and Cochran, 2010]. However, the success of periodontal regeneration by GTR depends on the experience or skills of an operator because of the high degree of technical difficulty of placing a barrier membrane under the gingiva. In contrast, EMD has been used for periodontal regenerative procedures through the promotion of cementum

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*Correspondence to: Takao Fukuda, Department of Periodontology, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3–1-1 Maidashi, Higashi-ku, Fukuoka, 812–8582, Japan.
E-mail: tfukuda@dent.kyushu-u.ac.jp

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development, and it appears to promote better clinical outcomes than open-flap debridement [Hammarström, 1997]. However, EMD is not as effective as GTR in terms of preserving space for new bone formation. Recently, the use of recombinant human basic fibroblast growth factor (bFGF) as a periodontal tissue regeneration reagent has been introduced. bFGF induces the proliferation of various cells, and the efficacy of local application of bFGF has been investigated in a clinical study [Kitamura et al., 2008, 2011]. It may also promote epithelial cell growth.

Sprouty (Spry) has been identified as an antagonist of the FGF receptor (FGFR) in tracheal and eye development in *Drosophila*, and *Drosophila* Spry inhibits FGF signaling by inhibiting the Ras-Raf1-mitogen-activated protein kinase (MAPK) pathway [Kramer et al., 1999; Kim and Bar-Sagi, 2004; Mason et al., 2006]. In mammals, four Spry have been identified, and Spry2, specifically, functions as a negative regulator of receptor tyrosine kinase (RTK) signaling [Gross et al., 2001; Impagntiello et al., 2001; Hanafusa et al., 2002; Yusoff et al., 2002]. Spry proteins have a short region of sequence similarity in their amino termini that contains a conserved tyrosine residue. In mammalian Spry2, this tyrosine residue is located at amino acid position 55 (Y55), and many of the inhibitory functions of Spry2 are dependent on this residue [Heldin, 1995; Guy et al., 2003, 2009]. Previous studies have suggested that a dominant-negative version of Spry2, in which the tyrosine residue had been mutated, enhanced fibroblast growth factor (FGF)- but not epidermal growth factor (EGF)-induced extracellular signal-regulated kinase (ERK) activation in 293 fibroblast cells [Sasaki et al., 2001]. Several studies have shown that *Spry2* knockout mice have supernumerary teeth in a normally toothless region called the diastema, indicating that *Spry2* functions to prevent development of teeth in the diastima by blocking FGF signaling [Goodnough et al., 2007; Lagronova-Churava et al., 2013]. Overexpression of *SPRY2* at the initiation of craniofacial development in chick embryos results in a dramatic disruption in the outgrowth of facial prominence, with cleft lip and palate [Klein et al., 2006]. Moreover, suppression of Spry2 and Spry4 expression in vivo accelerates angiogenesis and has a therapeutic effect in a mouse model of hind limb ischemia [Taniguchi et al., 2009]. In addition, it was also reported that Spry2 downregulation strongly promotes elongative axon growth by sensory neurons [Hausott et al., 2009].

Periodontal tissue consists of four principal components, namely the gingiva, alveolar bone, cementum, and periodontal ligament. With the exception of the gingiva, the principal components are derived from ectomesenchyme. At the earliest stages of tooth development, spherical condensation of ectomesenchyme forms to surround the developing dental organ. This surrounding spherical condensation is called the dental follicle, which will give rise to most of the alveolar bone, cementum, and periodontal ligament [Cho and Garant, 2000]. Regarding the relationship between mesenchymal cells and Spry2, it was reported that *Spry2* expression is an early response to stimulation by FGF1 in MC3T3-E1 osteoblastic cells and acts as a feedback inhibitor of FGF1-induced osteoblast responses [Yang et al., 2006]. However, the effects of Spry2 on osteoblast function remain unclear.

Here, we investigated how the inhibition of Spry2 affects the cellular physiology of mesenchymal cells (osteoblasts) and epithelial cells (gingival epithelial cells) in vitro. The present study revealed that suppression of *Spry2* expression induced proliferation and differentiation of osteoblastic cells upon bFGF and EGF stimulation, whereas it diminished proliferation of gingival epithelial cells. Our in vitro finding may support Spry2 as a novel therapeutic target for periodontal tissue regeneration.

MATERIALS AND METHODS

CELL CULTURE

MC3T3-E1 murine calvarial osteoblastic cells and GE1 mouse-derived gingival epithelial cells were purchased from RIKEN BioResouce Center (Ibaraki, Japan). MC3T3-E1 cells were cultured in α- minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in a 5% CO_2 incubator. GE1cells were cultured in α-MEM containing 1% FBS and 10 ng/ml EGF at 37 °C. Cells were serum-starved for 24 h, and then stimulated with 50 ng/ml recombinant bFGF (R&D Systems, Minneapolis, MN), 50 ng/ml recombinant EGF (R&D Systems), or 50 ng/ml each of both of bFGF and EGF as indicated in the figure legends.

TRANSIENT TRANSFECTION

The murine c-myc-Y55A-Spry2 dominant-negative vector and empty vector (gift from Dr. Yoshimura, Keio University of Medicine, Tokyo, Japan) have been described previously [Sasaki et al., 2001]. Cells were transfected with myc-Y55A-Spry2 or empty vector using the lipofection reagent LipofectAMINE and PLUSReagent (Invitrogen). Expression of the mutant mouse Spry2 protein in transfected cells was confirmed by Western blot analysis with the c-myc polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

WESTERN BLOT AND IMMUNOPRECIPITATION ANALYSES

Transfectants were lysed and cell extracts representing equal amount of total protein were separated by SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking in TBS-T buffer containing 5% (w/v) nonfat milk, the membrane was incubated with with either anti-mouse Spry2 (Upstate, Lake Placid, NY), anti-phospho-ERK, anti-ERK (Cell Signaling Technology, Danvers, MA), anti-runt-related transcription factor 2 (Runx2) (Santa Cruz Biotechnology), anti-twist related protein (Twist) (Santa Cruz Biotechnology), anti-FGFR1 (Cell Signaling), anti-EGF receptor (EGFR) (Cell Signaling), or c-myc antibodies at 4 °C overnight. As a control, β-action was detected using an anti-β-action antibody (Cell Signaling). The membrane was washed and exposed to horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using the ECL Western Blotting Substrate (Pierce, Rockford, IL). Densitometric analysis of bands was performed using the Image J program (NIH, Bethesda, MD). For immunoprecipitation analysis, cell extracts were incubated with protein G sepharose Glutathione Sepharose 4B (GE healthcare) conjugated with anti-EGFR or anti-c-Cbl antibodies (Cell Signaling). The precipitates were subject to Western blotting with anti-ubiquitin (Cell Signaling) or anti-Spry2 antibodies.

CELL PROLIFERATION ASSAY

Transfectants were placed in 96-well plates (5×10^4 cells/well) in triplicate. The cells were incubated with 50 ng/ml bFGF, 50 ng/ml EGF, or 50 ng/ml each of bFGF and EGF after 24 h post transfection. Then, WST-8 solution (Cell Count Reagent SF™: Nakarai Tesuque, Kyoto, Japan) was added to each well at 24, 48, and 72 h. After an additional 3 h of incubation at 37 °C, optical absorption at 450 nm was measured at 650 nm as the reference. For Ki-67 staining assay, cell proliferation was determined by the number of cells positive for the proliferation marker Ki-67 using anti- Ki-67 antibody (Thermo Scientific, Miami, FL). Alexa Fluor 488-conjugated antibody (Molecular Probes, Invitrogen, Carlsbad, CA) was utilized as secondary antibody. Cell nuclei were counterstained with Hoechst 33258 (10 μg/ml; Molecular Probes, Invitrogen, Carlsbad, CA) fluorescence staining. Cells were visualized under a Nikon A1 fluorescence microscope (Nikon, Tokyo, Japan).

RNA ISOLATION AND REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT–PCR) ANALYSIS

Total RNA was extracted from cells using ISOGEN (Nippon Gene), according to the manufacturer's instructions. cDNA was made by reverse transcription using PrimScript™ 1st strand cDNA Synthesis Kit (TAKARA). PCR was performed using Taq DNA polymerase (TAKARA) with the gene-specific primer pairs as follows: collagen type I (*Coll*), 5′-CGAAAGGTGAACCTGGTGAT-3′ (forward) and 5′-TCCAGCAATACCCTGAGGTC-3′ (reverse); osteopontin (*Opn*), 5′-CCATCTCAGAAGCAGAATCTCC-3′ (forward) and 5′-ATGGTCAT-CATCGTGGTCC-3′ (reverse); Osteocalcin (*Ocn*), 5′-TGAGAGCCCT-CACACTCCTC-3′ (forward) and 5′-TCAGCCAACTCGTCACAGTC-3′ (reverse); bone sialoprotein (*Bsp*), 5′-AATGGAGACGGCGATAGTTC-3′ (forward) and 5′-GTCCTCATAAGCTCGGTAAG-3′ (reverse); alkaline phosphatase (*Alp*), 5′-AACCCAGACACAAGCATTCC-3′ (forward) and 5′-GCCTTTGAGGTTTTTGGTCA-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5′-ATGGT-GAAGGTCGGTCAACGGA-3′ (forward) and 5′-ACTCCTTGGAGGC-CATGTAG-3′(reverse). PCR conditions were as follows: 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min; and a final step at 72 °C for 10 min. Electrophoresis was run for aliquot of PCR amplification products on a 2% (w/v) agarose gel, followed by detection of DNA with ethidium bromide. Densitometric analysis of bands was performed using the Image J program.

DETERMINATION OF ALKALINE PHOSPHATASE (ALP) ACTIVITY AND STAINING

For osteogenic differentiation, MC3T3-E1 cells transfected with Y55A-Spry2 at confluence were cultured in osteogenic medium consisting of α-MEM supplemented with 2 mM β-glycerophosphate (Wako, Osaka, Japan) and 50 μg/ml ascorbic acid (Wako). bFGF and EGF were added to a final concentration of 50 ng/ml each. ALP activities were measured using the ALP Assay Kit® (TAKARA, Shiga, Japan). The cells were placed in 24-well plates at a density of 5×10^3 cells/well. After washing with distilled water, the cells were lysed with 1% NP-40. Next, 0.2 M Tris-HCl (pH 9.5) containing 1 mM $MgCl_2$ were mixed with p-nitrophenyl phosphate as an ALP activities substrate, and were added to wells. The samples were incubated at 37 °C for 60 min, and 0.5 N NaOH was added to stop the reaction. The absorbance of each sample was measured at 405 nm using a microplate reader (Bio-Rad Laboratories). For ALP staining, cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde, followed by pretreatment with ALP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) and staining with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution.

IMMUNOFLUORESCENCE MICROSCOPY

GE1 cells transfected with Y55A-Spry2 were cultured on glass coverslips placed in 12-well tissue culture dishes in α-MEM containing 1% FBS for at least 24 h. The cells were cultured in medium with 50 ng/ml bFGF, 50 ng/ml EGF, or 50 ng/ml each of bFGF and EGF for 20 min at 37 °C, and then extensively washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 50 μg/ml digitonin, and blocked with 3% bovine serum albumin in PBS. Fluorescence detection of EGFR was performed with a mouse anti-EGFR antibody (Cell Signaling Technology) and anti-mouse Alexa Fluor 488 secondary antibody. Actin was stained with Alexa Fluor 594-conjugated phalloidin (Molecular Probes) and nuclei were stained with Hoechst 33258 (Molecular Probes). The coverslips were mounted using PermaFluor Mounting medium (Thermo Fisher Scientific, Waltham, MA). Cells were visualized under the Nikon A1 fluorescence microscope using 63 × /1.49 NA oil objectives. Images were obtained with the NIS-Elements AR 3.0 software, and the imaging parameters were kept constant whenever the intensity of fluorescence was to be compared.

STATISTICAL ANALYSIS

The data are the mean +/− standard deviation (SD) of an average of three samples and are representative of at least three distinct experiments. Statistical analyses were performed by unpaired Student's *t*-test or one-way ANOVA followed by Tukey post-hoc correction tests and a minimal level of $P < 0.05$ was considered significant.

RESULTS

Spry2 PROTEIN EXPRESSION AND ERK ACTIVATION IN MC3T3-E1 AND GE1 CELLS WITH EGF, bFGF, OR bFGF + EGF STIMULATION

To address whether Spry2 functions in MC3T3-E1 osteoblasts and GE1 gingival epithelial cells, we performed time course experiments and examined growth factor-induced Spry2 protein levels and ERK activation in those two cell types. When MC3T3-E1 cells and GE1 cells were stimulated with bFGF, EGF, or bFGF and EGF (bFGF + EGF), the Spry2 protein was constitutively expressed in both MC3T3-E1 and GE1 cells, regardless of the addition of growth factor (Fig. 1A,B). ERK activation peaked 10 min after the addition of recombinant human bFGF, EGF, or bFGF + EGF and then gradually decreased in MC3T3-E1 and GE1 cells (Fig. 1A,B). However, phosphorylation of ERK induced by bFGF was somewhat increased compared to that induced by EGF in MC3T3-E1 cells (Fig. 1A). On the other hand, we found EGF-induced ERK activation in GE1 cells (Fig. 1B). The strongest induction of ERK

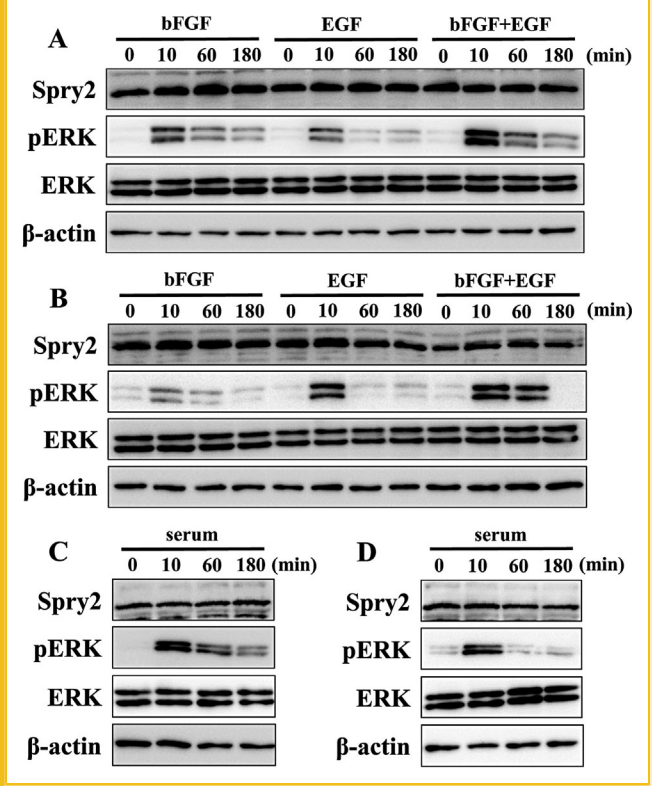

Fig. 1. Spry2 protein expression and ERK activation in MC3T3-E1 and GE1 cells stimulated with EGF, bFGF, or bFGF + EGF. Serum-starved MC3T3-E1 (A and C) and GE1 cells (B and D) (2×10^6 cells/well) were treated with bFGF (50 ng/ml), EGF (50 ng/ml), bFGF and EGF (50 ng/ml each), or 10% serum in six well plates for the indicated intervals of time. Cells were lysed, and equivalent amounts of protein were subjected to Western blot analysis. Separated bands were visualized using anti-mouse Spry2, anti-phospho-ERK and anti-ERK antibodies. As a control, β-actin was detected using anti-β-actin antibody. Results of one representative experiment out of three separate experiments are shown.

phosphorylation was observed when both cell types were stimulated with bFGF + EGF. Similar results were obtained when MC3T3-E1 and GE1 cells were stimulated with FBS (Fig. 1C,D). These results indicate that Spry2 proteins are constitutively expressed in osteoblasts and gingival epithelial cells without stimulation by bFGF, EGF, or bFGF + EGF, and upon stimulation by those factors, ERK phosphorylation is induced in MC3T3-E1 and GE1 cells.

Y55A-Spry2 INCREASES bFGF- AND EGF-INDUCED ERK ACTIVATION IN MC3T3-E1 CELLS BUT REDUCES IT IN GE1 CELLS

We next transfected a dominant-negative form of Spry2 (Y55A-Spry2) into MC3T3-E1 osteoblasts and GE1 gingival epithelial cells and then stimulated the cells with bFGF, EGF, or bFGF + EGF. In Y55A-Spry2, the tyrosine at position 55, which is essential for Spry2 function [Heldin, 1995; Guy et al., 2003, 2009], is substituted with alanine [Sasaki et al., 2001]. Transfection of Y55A-Spry2 increases bFGF-induced ERK activation but suppresses EGF-induced ERK activation in both MC3T3-E1 and GE1 cells (Fig. 2A,C). However, bFGF + EGF stimulation increases phosphorylation of ERK in Y55A-Spry2-transfected MC3T3-E1 cells (Fig. 2A,B). In contrast, we found that the Spry2 dominant-negative mutant decreased bFGF + EGF-induced ERK activation in GE1 cells (Fig. 2C,D). Moreover, results similar to stimulation by the combinatorial application of bFGF and EGF were obtained when Y55A-Spry2-transfected MC3T3-E1 and GE1 cells were stimulated with FBS (Fig. 2E,F). These results indicate that the Spry2 dominant-negative mutant increases bFGF + EGF-induced ERK activation in MC3T3-E1 cells but decreases it in GE1 cells.

Y55A-Spry2 INDUCED PROLIFERATION OF MC3T3-E1 CELLS AND REDUCED PROLIFERATION OF GE1 CELLS

We next focused on this interesting phenomenon of inversion by bFGF and EGF stimulation between two cell types transfected with Y55A-Spry2. To examine the proliferative effects of Y55A-Spry2 in osteoblastic or gingival epithelial cells, the WST-8 assay was performed on cells stimulated by bFGF + EGF. Consistent with the results described above (Fig. 2), Y55A-Spry2 enhances MC3T3-E1 cell proliferation (Fig. 3A) but significantly suppresses proliferation of GE1 cells (Fig. 3B) after bFGF + EGF stimulation. On the other hand, the difference between control- and Y55A-Spry2-transfected osteoblasts in the presence of serum only was not significant (Fig. 3A) although Y55A-Spry2 osteoblastic cells always grow slightly faster than control cells. In addition, Y55A-Spry2 increased the proliferation of MC3T3-E1 cells with bFGF stimulation (Fig. 3C), whereas it decreased the proliferative effect of both cells with EGF stimulation (Fig. 3C,D). We also analyzed the potential effects of Y55A-Spry2 on proliferation of the two cell types by using the cell proliferation marker, Ki-67, which is not expressed in G_0 phase. Transfection of Y55A-Spry2 caused an increase in the number of Ki-67 positive MC3T3-E1 cells after bFGF + EGF stimulation (Fig. 3E), whereas it decreased the number of Ki-67 positive GE1 cells (Fig. 3F). We confirmed that the Spry2dominant-negative mutant significantly induced cell proliferation in MC3T3-E1 cells but not in GE1 cells when they were stimulated with bFGF + EGF.

INHIBITION OF Spry2 INDUCES OSTEOBLASTIC DIFFERENTIATION IN MC3T3-E1 CELLS

Next, we cultured Y55A-Spry2 transfected MC3T3-E1 cells in osteogenic media in the presence of bFGF + EGF and examined the expression of osteoblast-specific genes. Alkaline phosphatase (*Alp*) and osteocalcin (*Ocn*) mRNA expression was upregulated in Y55A-Spry2 cells, whereas collagen type I (*Col1*), osteopontin (*Opn*) and bone sialoprotein (*Bsp*) mRNA expression levels were unchanged, as revealed by RT-PCR analysis (Fig. 4A,B). Additionally, ALP activity substantially increased when Y55A-Spry2-transfected MC3T3-E1 cells were grown in osteogenic media supplemented with EGF or bFGF + EGF for 3 days (Fig. 4C). Consistent with this result, the addition of EGF or bFGF + EGF to Y55A-Spry2-expressing cells led to increased ALP staining. In contrast, no significant difference in ALP staining was seen following bFGF, EGF, or bFGF + EGF treatment of cells transfected with empty vector (Fig. 4D). *Runx2* is a key regulator of osteoblast differentiation and is known to upregulate other genes important for later stages of osteoblast differentiation, and *Alp* and *Ocn* mRNA expression are controlled by Runx2 [Nakamura et al., 2010; Komori, 2011]. Interestingly, the level

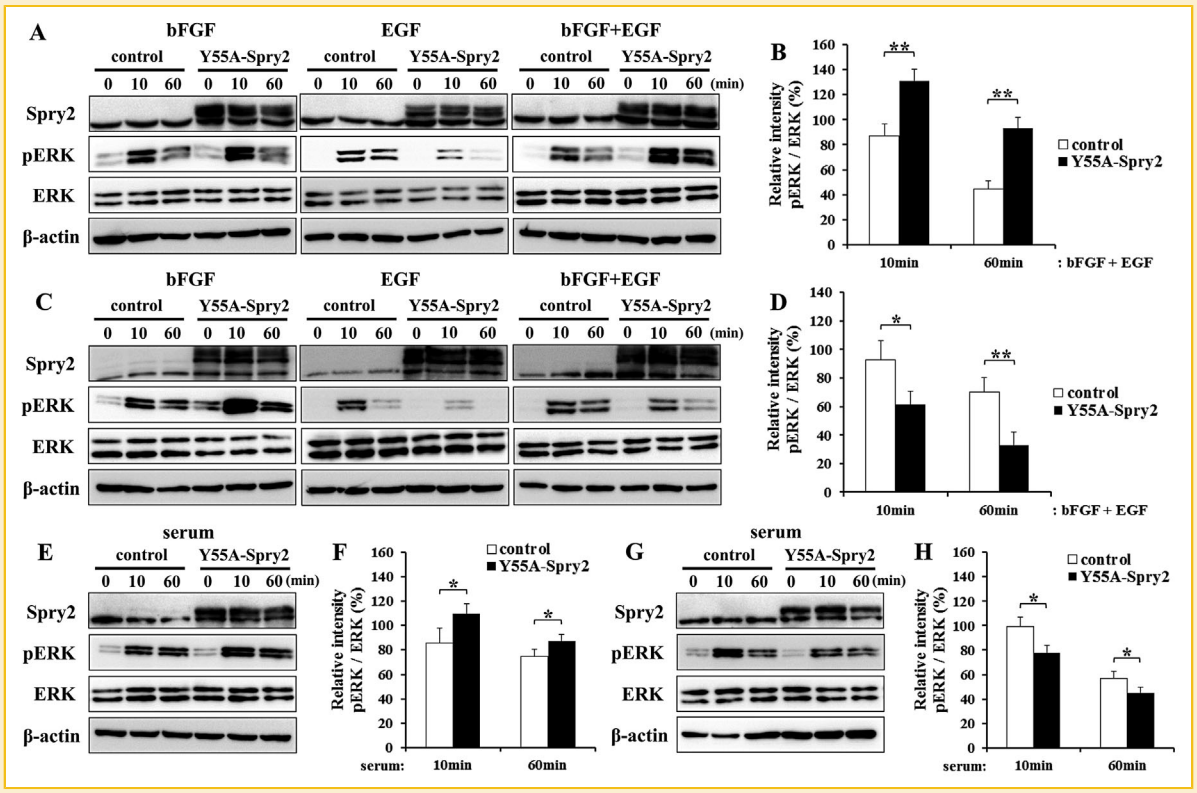

Fig. 2. Y55A-Spry2 increases bFGF and EGF-induced ERK activation in MC3T3-E1 cells but reduces it in GE1 cells. MC3T3-E1 osteoblastic cells (A, B, E and F) and GE1 gingival epithelial cells (C, D, G and H) were transduced with c-myc-Y55A Spry2 or a control vector. After 24 h, serum-starved cells were treated with 50 ng/ml bFGF, 50 ng/ml EGF, or 50 ng/ml each of bFGF and EGF, or 10% serum for the indicated times. Cell extracts were then resolved by SDS-PAGE and immunoblotted with anti-Spry2, anti-pERK and anti-ERK antibodies. As a control, β-actin was detected using anti-β-actin antibody. A, C, E and G: Representative Western blot analysis of pERK and total ERK protein levels using total protein from control and Y55A-Spry2 cells. B, D, F and H: Quantification analysis showing pERK protein level to total ERK in bFGF and EGF treated cells using the Image J program. The significance of differences between groups was determined by a two-tailed unpaired Student's test: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments.

of Runx2 protein in Y55A-Spry2 cells stimulated with bFGF, EGF, or bFGF + EGF increased significantly as revealed by Western blot analysis, whereas Runx2 expression in control cells was inhibited by EGF or bFGF + EGF stimulation (Fig. 4E,F). The twist basic helix-loop-helix transcription factor (Twist) also plays an important role in bone and cranial suture development, and is expressed primarily in osteoblastic cells [Murray et al., 1992] and preosteoblasts [Rice et al., 2000]. Twist is an upstream regulator of Runx2 that functions to downregulate Runx2 expression and prevent terminal osteoblastic differentiation [Singh et al., 2011]. Western blot (Fig. 4E,F) data confirmed an increase in Runx2 protein expression in Y55A-Spry2 osteoblastic cells treated with bFGF + EGF, whereas Twist expression was decreased. These data suggest that the Spry2 dominant-negative mutant decreases Twist expression, enhances Runx2 expression via EGFR signaling, and induces osteoblastogenesis by activating ALP activity and osteocalcin expression.

Y55A-Spry2 INDUCES UBIQUITINATION AND PROTEASOMAL DEGRADATION OF EGF RECEPTORS IN GE1 CELLS TO DOWNREGULATE EGF RECEPTOR EXPRESSION

We found that cell proliferation in Y55A-Spry2-transfected gingival epithelial cells was inhibited by EGF or bFGF + EGF stimulation compared to the control cells (Fig. 3B,D,F). Therefore, to identify the possible causative mechanism, we examined FGFR and EGFR expression after bFGF, EGF, or bFGF + EGF stimulation in Y55A-Spry2-transfected GE1 cells by Western blot. Y55A-Spry2 decreases the level of EGFR protein in GE1 cells (Fig. 5B), although the level of FGFR expression in Y55A-Spry2 cells is not different from that in the control cells (Fig. 5A). Similar results were obtained when Y55A-Spry2-transfected MC3T3-E1 osteoblasts were stimulated by bFGF + EGF (Fig. 5C,D). As the cell membrane expression of activated tyrosine kinase receptors is regulated by negative feedback mechanisms involving receptor degradation, we hypothesized that downregulation of the EGFR in Y55A-Spry2-transfected GE1 cells could be due to enhanced degradation of this receptor. To test this hypothesis, we examined the interaction of EGFR with ubiquitin. Immunoprecipitation analysis showed that the level of ubiquitin-associated EGFR was much higher in Y55A-Spry2-transfected GE1 cells than in control cells (Fig. 5E). Additionally, to investigate the role of the endogenous Spry2 for the increased ubiquitination of EGFR in presence of Y55A-Spry2, we performed immunoprecipitation method using an anti-c-Cbl antibody. We found that Y55A-Spry2 decreased endogenous Spry2 bound to c-Cbl, an E3 ubiquitin ligase, when GE1 cells were stimulated with EGF or bFGF + EGF

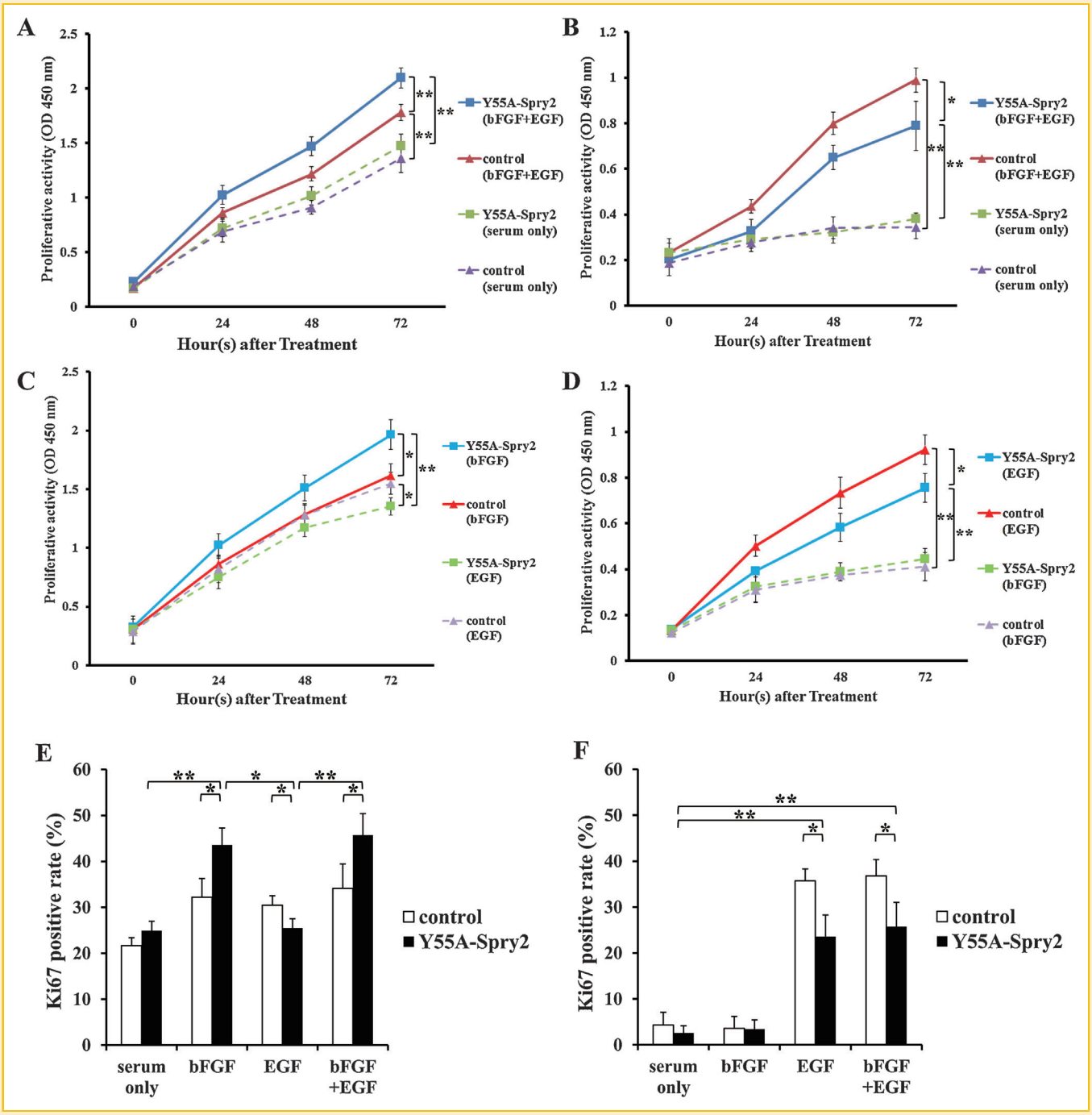

Fig. 3. Cell proliferation was induced by Y55A-Spry2 in MC3T3-E1, whereas it was reduced in GE1 cells. Proliferation activities of MC3T3-E1 (A and C) and GE1 (B and D) cells transfected with Y55A-Spry2 or control vector were measured by the WST-8 assay. After 24 h, cells were incubated in a-MEM containing 10% FBS with or without the addition of 50 ng/ml bFGF, 50 ng/ml EGF, or 50 ng/ml each of bFGF and EGF. Proliferation is expressed as the absorbance at 450–655 nm. Cell proliferation of MC3T3-E1 (E) and GE1 (F) cells transfected with Y55A-Spry2 or the control was also determined by the number of cells positive for Ki-67. The significance of differences between groups was determined by one-way ANOVA/Tukey: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments.

(Fig. 5F). Next, we observed the subcellular localization of EGFR in Y55A-Spry2-transfected GE1 cells. Endocytosed EGFR was distributed throughout the cytoplasm in EGF- or bFGF + EGF-stimulated control cells, but the staining was decreased in GE1 cells transfected with Y55A-Spry2 (Fig. 5G–I). These data indicate that Spry2 sequestration in GE1 cells results in the reduction of EGFR expression via ubiquitination and degradation without recycling of endocytosed receptors.

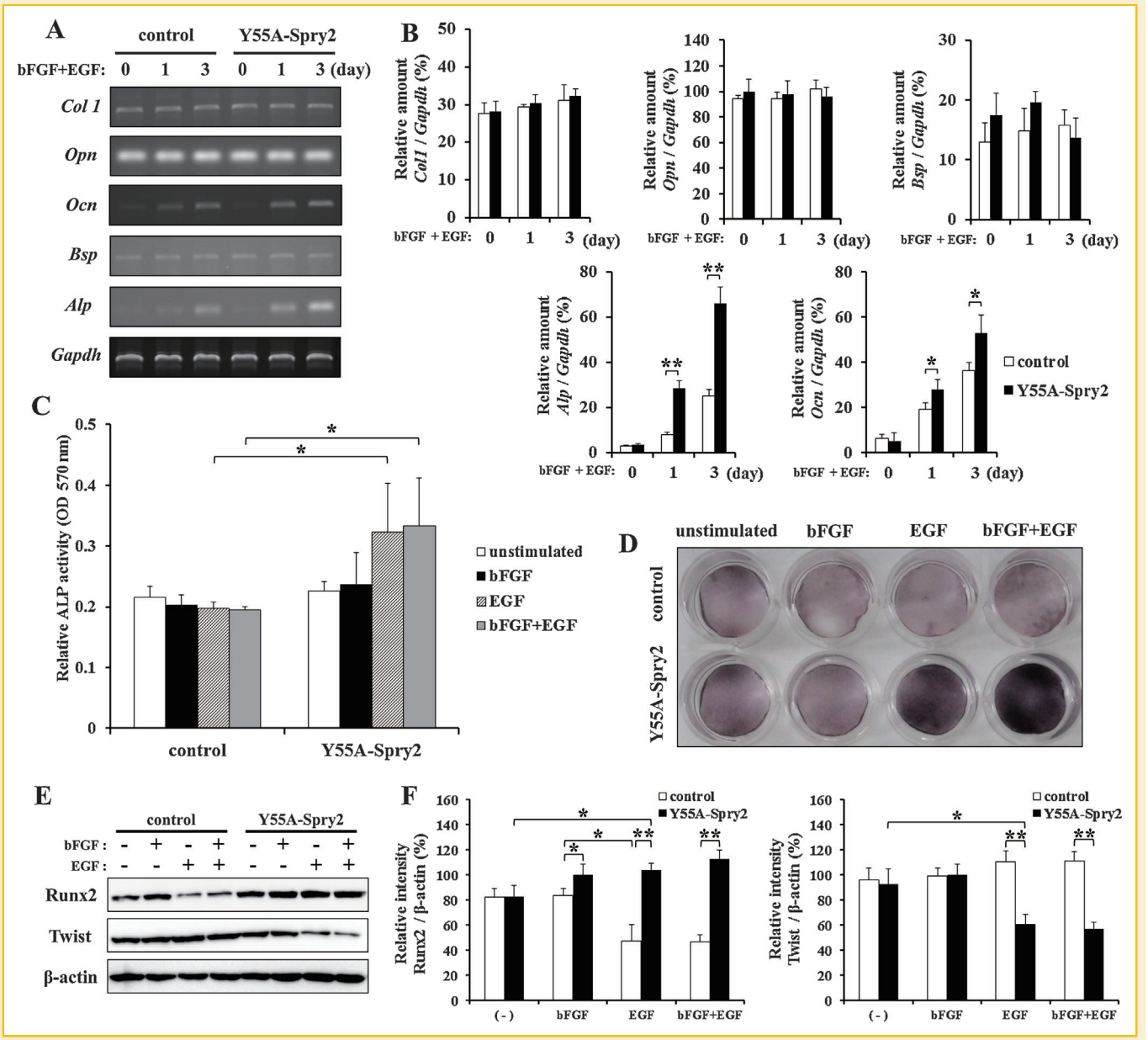

Fig. 4. Inhibition of Spry2 induced osteoblastic differentiation in MC3T3-E1 cells. A: Representative RT-PCR of osteoblastic marker genes mRNA expression levels. Expression of osteoblastic marker genes in Y55A-Spry2-transfected MC3T3-E1 cells. Transfectants were cultured in osteogenic media in the absence or the presence of 50 ng/ml each of bFGF + EGF, and the mRNA expression of the indicated genes was monitored by RT-PCR at the indicated time. *Coll*, collagen type I; *Opn*, osteopontin; *Ocn*, osteocalcin; *Bsp*, bone sialoprotein; *Alp*, alkaline phosphatase. *Gapdh* was used as a control. B: Quantification of mRNA expression of osteoblastic marker genes mRNA expression using the Image J program. The significance of differences between groups was determined by a two-tailed unpaired Student's test: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments. C: Transfected osteoblastic cells were cultured in osteogenic media with the addition of 50 ng/ml bFGF, 50 ng/ml EGF, or 50 ng/ml each of bFGF + EGF for 3 days, and ALP activity during the differentiation was measured. ALP activity is expressed as the absorbance at 570–655 nm. The significance of differences between groups was determined by one-way ANOVA/Tukey: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments. D: MC3T3-E1 cells transfected with the control vector or Y55A-Spry2 were seeded on 24-well plates. After confirming the confluence of each well, the cells were continuously incubated in osteogenic media with the addition of bFGF, EGF, or bFGF + EGF for 3 days. ALP staining was performed after osteogenic stimulation. Similar results were obtained in three independent experiments, and representative data are shown. E: Representative Western blot analysis of Runx2 and Twist protein levels from control and Y55A-Spry2 cells. MC3T3-E1 cells transfected with control or Y55A-Spry2 vector were incubated in osteogenic media with bFGF, EGF, or bFGF + EGF for 3 days. Total cell lysate was harvested and protein expression of Runx2 and Twist was analyzed by Western blotting. β-actin was used as a loading control. F: Protein expression was quantified using the Image J program. The significance of differences between groups was determined by one-way ANOVA/Tukey: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments.

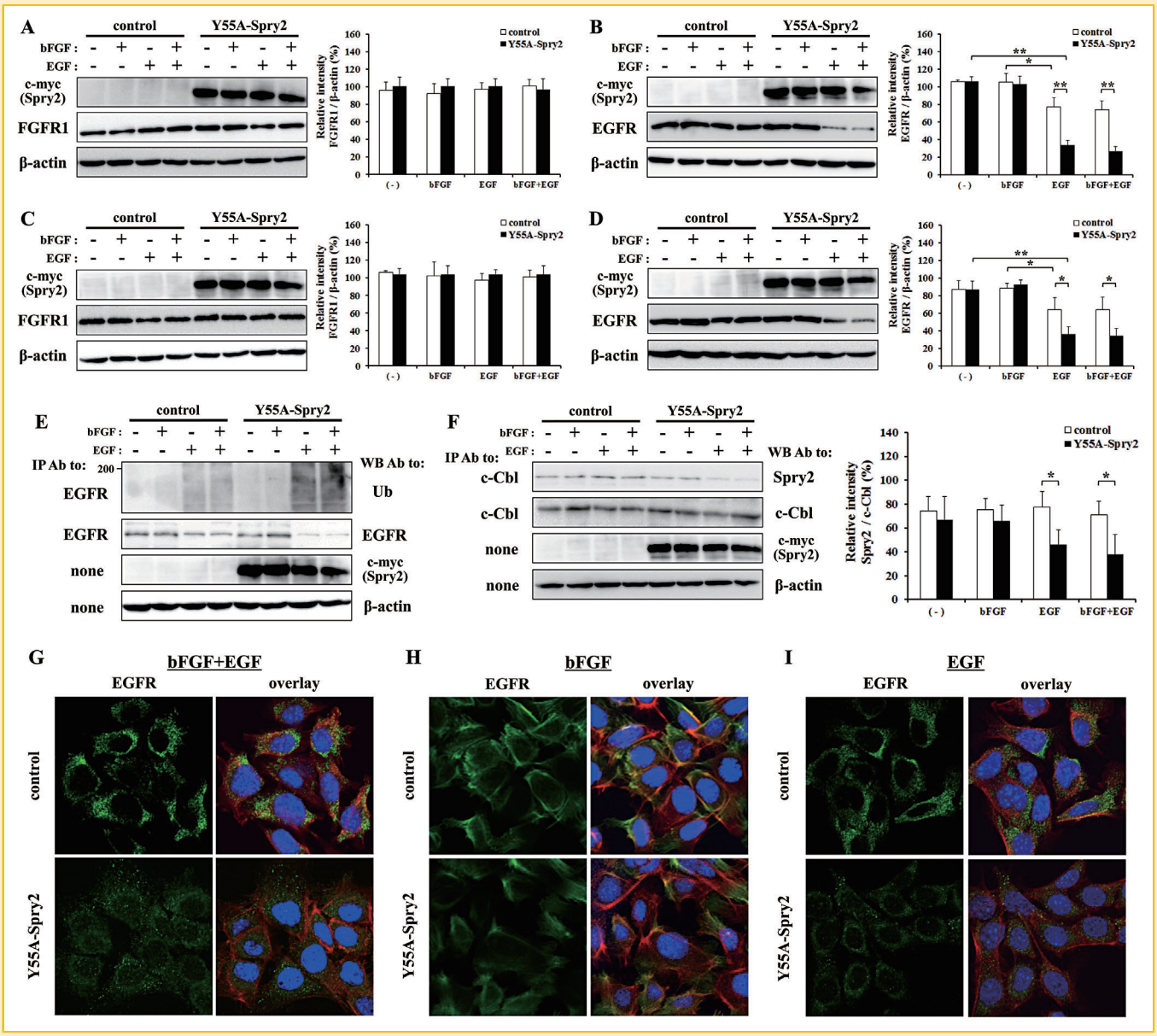

Fig. 5. Y55A-Spry2 induced ubiquitination and proteasomal degradation of EGF receptors in GE1 cells with decreased EGFR expression. After stimulation by 50 ng/ml bFGF, 50 ng/ml EGF, and 50 ng/ml each of bFGF and EGF for 20 min, whole cell lysates prepared from GE1 cells (A and B) or MC3T3-E1 cells (C and D) transfected with Y55A-Spry2 or control vector were immunoblotted with anti-FGFR1 (A and C) and anti-EGFR (B and D), using anti-β-actin as control. Protein expression was quantified using the Image J program. The significance of differences between groups was determined by one-way ANOVA/Tukey: $^{*}P < 0.05$; $^{**}P < 0.01$. Data represent mean ± SD. Similar results were obtained in three independent experiments. E, F: Whole cell lysates from GE1 cells transfected with Y55A-Spry2 or control vector were immunoprecipitated with anti-EGFR (E) or anti-c-Cbl (F) and the proteins were immunoreacted with anti-ubiquitin, or anti-EGFR antibodies (E) or anti-Spry2, or anti-c-Cbl antibodies (F). IP, immunoprecipitation; WB, Western blot. G–I: After transfection with Y55A-Spry2 or control vector, GE1 cells were incubated with 50 ng/ml each of bFGF and EGF (G), 50ng/ml bFGF (H), and 50 ng/ml EGF (I) at 37 °C for 20 min. For fluorescence microscopy, the cells were fixed, permeabilized, and stained with the EGFR antibody, followed by an Alexa 488 secondary antibody (green). F-actin was stained with Alexa Fluor 594 phalloidin (red). Nuclei were stained with Hoechst dye (blue). Cells were visualized under the Nikon A1 fluorescence microscope using 63 × /1.49 NA oil objectives. Images were obtained with the NIS-Elements AR 3.0 software, and the imaging parameters were kept constant whenever the intensity of fluorescence was to be compared. All confocal images are representatives of experiments conducted in triplicates.

DISCUSSION

Our study showed, for the first time, that Y55 mutation of Spry2 increased bFGF + EGF-induced ERK activation in MC3T3-E1 osteoblastic cells but decreased it in GE1 gingival epithelial cells. Consistent with this observation, a Spry2 dominant-negative mutant increased osteoblast proliferation but suppressed GE1 cell proliferation.

Spry2 is tyrosine-phosphorylated on a conserved N-terminal tyrosine residue (Y55) in response to RTK stimulation and subsequently interacts with Grb2, c-Cbl, Raf1, Shp2, and FRS2 [Guy et al., 2003; Kim and Bar-Sagi, 2004]. The interaction of

Spry2 with Grb2 and c-Cbl has been shown to be phosphotyrosine-dependent. Spry2 associates with the Src-homolog 2 (SH2) domain of Grb2, an adaptor molecule that connects RTK activation to the Ras/Raf1 pathway, after FGFR-induced phosphorylation of Y55. As a result, the interaction of Grb2 with FGFR adaptor molecules, FRS2 or Shp2, is blocked and FGF-induced signal transduction is inhibited [Hanafusa et al., 2002; Christofori, 2003], although the regulatory mechanisms underlying this inhibition remains inconclusive. Other studies have reported that cellular activation by EGF induces Y55-phosphorylation of Spry2 and enhances binding to c-Cbl, an E3 ubiquitin ligase. The binding of Spry2 to c-Cbl results in the sequestration of c-Cbl, preventing it from binding to EGFR, which results in reduced EGFR ubiquitination and degradation and thus, sustained EGFR activation [Wong et al., 2002; Rubin et al., 2003]. Here, we showed that treatment of Y55A-Spry2 transfected MC3T3-E1 cells with a bFGF and EGF cocktail led to increased proliferation compared with control cells. The Y55A-Spry2 dominant-negative mutant is thought to function by forming heterodimers with endogenous Spry2 and interfering with the binding of endogenous Spry2 to its targets in the MAPK pathways, thereby promoting the opposite phenotype of the Spry2 protein [Sasaki et al., 2001; Hanafusa et al., 2002]. Y55A-Spry2 may enhance FGFR signaling by preventing the interaction between Grb2 and endogenous Spry2 and may attenuate EGFR signaling by inducing the c-Cbl-mediated ubiquitination and degradation of EGFR in both cells (Fig. 6). Proliferative upregulation in Y55A-Spry2-transfected MC3T3-E1 cells that have been simultaneously stimulated by FGFR and EGFR may be caused by the more qualitative or quantitative superiority of FGFR signaling than EGFR signaling in osteoblasts (Fig. 6B). Overexpression of Spry2 has been shown to inhibit cell proliferation in human sarcoma cells [Lee et al., 2004] and osteosarcoma-derived cells [Rathmanner et al., 2013], whereas downregulation of Spry2 enhances proliferative potential in oral palate mesenchymal cells [Matsumura et al., 2011]. Conversely, GE1 gingival epithelial cells depend on EGFR signaling for cell proliferation because these cells are unable to survive in culture media that has not been supplemented with EGF. Therefore, our results suggest that Y55A-Spry2 enhances the ubiquitination and degradation of EGFR; consequently, EGFR expression is reduced, thereby decreasing cell proliferation, counteracting the proliferative increase that would be induced by enhanced FGFR signaling in GE1 cells (Fig. 6A). In addition, our results indicated that Y55A-Spry2 prevented endogenous Spry2 from binding to c-Cbl when gingival epithelial cells were stimulated with EGF or bFGF + EGF (Fig. 5F), whereas earlier reports have shown that Spry2 dominant-negative mutant unable to bind c-Cbl did not function as dominant-negative form and kept EGFR levels unchanged [Fong et al., 2003; Haglund et al., 2005]. Although the precise mechanism through which Y55A-Spry2 suppresses endogenous Spry2 and c-Cbl interaction remains unclear, it was recently reported that intersectin1 (ITSN1), which stimulates ubiquination of EGFR through enhancing the activity of Cbl, interacts with and recruits the Shp2 tyrosine phosphatase to Spry2 to enhance its dephosphorylation, thereby disrupting the inhibitory effect of Spry2 on Cbl and increasing EGFR ubiquitination [Okur et al., 2014]. While our results indicate that Y55A-Spry2 enhances EGFR ubiquitination and degradation, our data do not address the functional consequences of this Spry2- Cbl complex and Y55A-Spry2 interaction. A number of kinases, two ubiquitin E3 ligases and several phosphatases are

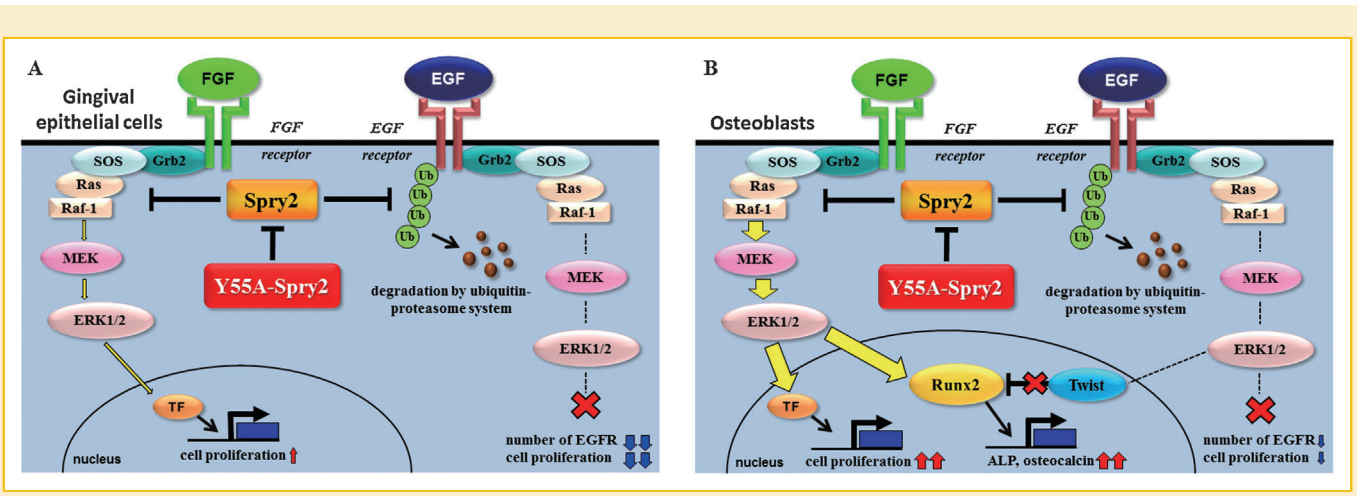

Fig. 6. Proposed model of the involvement of Spry2 and Y55A-Spry2 between FGF and EGF signaling for cell proliferation in gingival epithelial cells (A) or cell proliferation and osteogenic differentiation in osteoblasts (B). Upon FGF and EGF binding to their respective receptors and activating the respective signal pathways, Spry2 becomes activated and interacts with various components of the MAPK signaling pathway. The dominant-negative Y55A-Spry2 inhibits endogenous Spry2, thereby promoting the FGF-induced Raf/MEK/ERK pathway or suppressing the EGF-induced Raf/MEK/ERK pathway. A: Y55A-Spry2 enhances the ubiquitination and degradation of EGFR, which leads to a reduction in EGFR expression and decreases cell proliferation in gingival epithelial cells. B: FGF stimulation increases cell proliferation and Runx2 expression via the ERK signaling pathway and promotes FGF-stimulated osteogenic differentiation in osteoblasts. In addition, the suppression of EGFR signaling due to increased degradation causes Runx2 activity to increase through the inhibition of Twist, a negative regulator of Runx2. Y55A-Spry2 in osteoblasts induces both cell proliferation and cell differentiation by FGF + EGF-stimulated ERK phosphorylation. Ub, ubiquitin; TF, transcription factor.

reported as the assortment of protein types which are interacting with Spry2 [Guy et al., 2009]. Thus, further detailed studies to define a complex network of interactions among Y55A-Spry2, endogenous Spry2, c-Cbl, Shp2, ITSN1, and so on during ubiquitination process will be needed.

We also found that not only cell proliferation, but also cell differentiation, is increased in Spry2 dominant-negative mutant osteoblasts, at levels far beyond our expectations. This correlates with the upregulation of Runx2 expression and the downregulation of Twist expression by Y55A-Spry2 in osteoblasts (Fig. 4E, F). Runx2 is a master osteogenic regulator and functions as an inducer and regulator of osteoblastic differentiation by regulating the expression of numerous osteoblast-specific genes [Komori, 2011]. Twist is negatively involved in osteoblastic differentiation by interfering with Runx2 function at early stages of osteogenesis [Lee et al., 1999]. Moreover, Runx2 is related to ERK activation and phosphorylation, which enhances osteoblast gene expression in the bone environment [Xiao et al., 2002]. bFGF stimulation increases ERK signaling via FGFR, ERK-activated Runx2, and ALP expression; in addition, it suppressed Twist expression, thus stimulating bFGF-stimulated osteogenic differentiation in giant cell tumor osteoblast-like stromal cells [Singh et al., 2012]. Consistent with these previous published reports, our results indicated that bFGF stimulation increases cell proliferation and Runx2 expression via the ERK signaling pathway and promotes bFGF-stimulated osteogenic differentiation in Y55A-Spry2 transfected osteoblasts (Fig. 6B). On the other hand, EGF-EGFR signaling inhibits the differentiation of osteoblasts by suppression of Runx2 activity, and these effects are Ras-dependent; in addition, ERK has been shown to modulate Runx2 activity through the localization of Smad1 and the induction of Twist [Nakamura et al., 2010; Zhu et al., 2011]. The results presented in this article demonstrated that a reduction of Spry2 activity in MC3T3-E1 cells induced both cell proliferation and differentiation by bFGF + EGF-stimulated ERK phosphorylation and that Y55A-Spry2 upregulated Ras-responsive Runx2 expression, suggesting that Y55A-Spry2 may regulate Runx2 expression in MC3T3-E1 cells by inducing the activation of FGF stimulation and the degradation of EGFR. In other words, the enhanced differentiation observed in Y55A-Spry2 transfected osteoblastic cells may be caused by enhancing Runx2 upregulation induced by FGFR signaling, blocking EGFR signaling, and relieving the inhibition of Runx2 by Twist (Fig. 6B). A previous study also indicated that overexpression of Spry2 in NIH3T3 and PC12 cells can inhibit both cell proliferation and differentiation by preventing Ras activation [Gross et al., 2001]. In addition, an earlier report showed that transient overexpression of Spry2 in serum-starved MC3T3-E1 cells decreased acidic FGF (aFGF)-mediated ERK phosphorylation and aFGF-stimulated Opn promoter-driven luciferase activity [Yang et al., 2006]. In contrast, our findings revealed that Spry2 dominant-negative mutant upregulated *Alp* (mature osteoblast marker) and *Ocn* (a late osteoblastic marker) mRNA expression in MC3T3-E1cells cultured in osteogenic medium, while *Opn* (an early osteoblastic marker) mRNA expression levels were almost the same throughout the experiments (Fig. 4A, B). Our data are different from the earlier report in many ways (e.g., Spry2 wild-type vector or dominant-negative vector; aFGF or bFGF + EGF; promoter activity or mRNA expression; or cell culture methods before stimulation). Moreover, it has been shown that Opn mRNA expression in MC3T3-E1 pre-osteoblastic cells at higher passage number was elevated compared with that in cells at lower passage number [Huang et al., 2004]. Therefore, a large amount of expression of *Opn* mRNA and no significant difference of expression between control and Y55A-Spry2 in Figure 4A may be caused by high passage of MC3T3-E1 cells.

Interestingly, it was recently reported that Spry2 and Spry4 are physiologically important negative regulators of angiogenesis in vivo and suppressing them has a novel therapeutic effect for treating peripheral ischemic diseases [Taniguchi et al., 2009]. In addition, the dominant-negative Spry2 activates proliferation, migration, and differentiation of endothelial cells, promoting mouse skin wound healing [Wietecha et al., 2011]. Furthermore, Spry2 RNAi strongly promotes elongative axon growth of sensory neurons [Hausott et al., 2009]. Thus, the suppression of *Spry2* could benefit tissue regeneration by enhancing the stimulation of bFGF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF). Periodontal tissue regeneration requires new dental cementum and periodontal ligament formation, aside from the formation of new bone, in addition to the prevention of gingival epithelial cell downgrowth. Therefore, whether Spry2 inhibition, together with treatment by bFGF and EGF is linked to regeneration of the cementum and periodontal ligament is an important issue that should be further investigated in vivo in the future.

In conclusion, we found that tyrosine 55 mutation of Spry2 induced proliferation and differentiation of osteoblastic cells by bFGF and EGF stimulation, whereas it diminished cell proliferation of gingival epithelial cells, implicating a tyrosine residue at amino acid position 55 of Spry2 as a novel therapeutic target for periodontal tissue regeneration. These in vitro experiments may provide a molecular basis for novel therapeutic approaches in periodontal tissue regeneration. In other words, the combined application of a Spry2 inhibitor, bFGF, and EGF may effectively allow alveolar bone to grow and block the ingrowth of gingival epithelial cells in bony defects, biologically mimicking the barrier effect seen in conventional GTR, and this has potential for development as a new regenerative strategy.

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