# Regulation of NF-kB Signalling Through the PR55β-RelA Interaction in Osteoblasts

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**Abstract.** *Background/Aim: Nuclear factor kappa B (NF-kB) signalling including the RelA subunit is activated upon fibroblast growth factor (FGF) stimulation. A clear understanding of the mechanisms underlying this action will provide insights into molecular targeting therapy. Furthermore, protein phosphatase 2A (PP2A) is involved in RelA dephosphorylation, but little is known about the underlying mechanism. Materials and Methods: Because the regulatory subunits of PP2A drive NF-kB signalling via RelA, we used qRT-PCR and immunoblot analysis to investigate the expression of these subunits in MC3T3-E1 cells. We examined weather FGF2 interacts with NF-kB using immunocytochemistry (IC), immunoprecipitation (IP), and pull-down assay (PD) using recombinant proteins. Results: PR55β expression was increased, whereas activated RelA was dephosphorylated upon FGF2 stimulation. Further, the interaction of PR55β with RelA was confirmed by IC, IP, and PD. Conclusion: FGF2-induced PR55β directly interacts with RelA and regulates NF-kB signalling.*

Bone metabolism is impaired by severe inflammation, which results in osteomyelitis, especially in the jaw (1). In the bone, osteoblasts are critical for the defensive response as they induce cytokine release to strongly support suppression of osteolysis and promotion of new bone formation (2). Accordingly, there has been a recent increase in the number of studies focusing on the crosstalk between inflammatory

signals and growth factors such as fibroblast growth factors (FGFs) (3). However, a deeper understanding of the regulatory mechanisms is necessary to develop molecular targeting therapies for bone inflammation.

FGFs have multiple roles as in patterning, morphogenesis, proliferation, differentiation, and cell migration (4). Moreover, increasing evidence suggests a direct chemotactic response of osteoblasts to FGF signals (5). On the contrary, FGFs are known to be involved in inflammatory signalling. The vast majority of inflammation mainly involves nuclear factor kappa B (NF-kB) signalling, indicating that regulation of its activation by FGFs is required for bone homeostasis (6). Multiple FGFs bind to and activate multiple FGFRs, which are tyrosine kinase receptors containing three immunoglobulin-like domains and a heparin-binding sequence (7). Interactions between these molecules increase the rates of cell proliferation and differentiation. In particular, FGF2, a main FGF signalling ligand, is reported as an activator of FGFRs in osteoblasts (8). However, negative regulation of FGF signals also occurs through activation of protein phosphatases, which is considered important for the interaction between the involved signals and FGF signals (9).

NF-kB is an important transcriptional factor that regulates many cellular and organismal processes including immune and inflammatory responses, cellular growth, and survival (10). NF-kB is a complex composed of two subunits designated p50 and RelA/p65, and is activated by several inflammatory cytokines including TNFα, which leads to IKK activation (11). In turn, IKK phosphorylates IkB proteins. Phosphorylation-induced degradation of IkB proteins allows NF-kB nuclear translocation and activation of transcription. Though NF-kB is phosphorylated by several kinases, protein phosphatases dephosphorylate each subunit. As an essential subunit for NF-kB activation and translocation, RelA/p65 is activated by the inhibitor of protein phosphate 2A (PP2A), indicating the involvement of PP2A in dephosphorylation (12). However, the mechanism of the underlying interaction is unclear.

PP2A is a major phosphatase that comprises a catalytic subunit (PP2Ac), a structural subunit (PR65/A), and a variable regulatory B subunit belonging to one of four classes (PR/B, PR/B', PR/B'', or PR/B''') (13). The substrate specificity of the holoenzyme is determined by the subcellular locale to which the complex is confined, selective incorporation of the B subunit, interactions with endogenous inhibitory proteins, and specific intermolecular interactions between PP2A and target substrates (13). Mammalian cytosolic PP2A is ubiquitously expressed and is implicated in almost every signalling pathway, including those that regulate bone metabolism (14). PP2A suppresses the activation of NF-kB signalling *via* dephosphorylation of P65/RelA (15). Although PP2A has also been reported to play a bone-promoting role, the mechanism underlying this effect remains poorly understood.

Further, although certain B subunits of PP2A are associated with osteoblasts, their expression levels and roles are not yet clear. In this study, we investigated the expression levels of different B subunits of PP2A in MC3T3-E1 osteoblastic cells, as well as the role of FGF2 in RelA dephosphorylation for NF-kB signalling.

## Materials and Methods

*Antibodies and reagents*. Recombinant murine FGF2 was obtained from R&D Systems (Minneapolis, MN, USA), and recombinant murine TNFα was obtained from Peprotech (Rocky Hill, NJ, USA). PD161570, a specific FGFR antagonist, was obtained from Tocris Bioscience (Boston, MA, USA). Anti-PR55β antibody was obtained from Abcam (Cambridge, MA, USA). Anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies, anti-phosphorylated NF-kB p65/RelA and anti-p65/RelA antibodies were obtained from Cell Signalling Technology (Danvers, MA, USA), and anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

*Cell culture and transfection*. MC3T3-E1 cells were supplied by the RIKEN cell bank. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a humidified 5% $CO_2$ incubator. The cells were transfected with siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After incubation, the medium was replaced. We used FGF2 (30 ng/μl) or TNFα (20 ng/μl) as the stimulation reagent with or without PD161570 (10 nM).

*RNA interference*. siRNA duplexes against murine *Ppp2r2b* and control (scrambled) siRNA were synthesised by Eurofins Genomics (Ebersberg, Germany). The sense strands of the siRNAs were as follows: negative control, 5'-AAUUCUCCGAACGUGUCACGU-3'; si*Ppp2r2b*, 5'-ACUUUCCACAGCUUCACAGTT-3'.

*Total RNA isolation*. Total RNA was extracted from MC3T3-E1 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA) was used as reverse transcriptase, and first-strand cDNA was synthesised from 3 μg of total RNA and random primers.

*Preparation of recombinant proteins*. GST-tagged recombinant proteins were purified from *Escherichia coli* BL-21 (DE3) transformed with appropriate plasmids. Culture medium was incubated to 0.4 absorbance at 600 nm at 37°C and then with 250 μM isopropyl β-D-1-thiogalactopyranoside at 18°C for an additional 12-14 h. Bacterial lysate was prepared by sonication in lysis buffer (50 mM Tris–HCl at pH 8.0, 300 mM NaCl, 25 μg/ml p-amidinophenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 5 ug/ml leupeptin and 2.5 μg/ml pepstatin). After the addition of 1% Triton X-100, rotation of the lysate was performed on for 30 min. Purification was achieved using an affinity chromatography resin, glutathione sepharose 4B (GE Healthcare, Buckinghamshire, UK). Purity was checked by staining with Coomassie Brilliant Blue after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were dialyzed with the assay solution, and centrifuged before use.

*Quantitative real-time RT-PCR*. All PCRs were performed using the Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). The following primer sets were used: *Ppp2r2a*, F: 5'-GCAACAGGAGATAAAGGTGGTAG-3', R: 5'-TGGTTCATGGCTCTGGAAGGTG-3'; *Ppp2r2b*, F: 5'-GCGTG ATAAGAGGCCAGAAG-3', R: 5'-TGTGTGCGTTGGCAAATACT-3'; *Ppp2r2c*, F: 5'-AGAGCTGATGACCTCACCGTTGTT-3', R: 5'-ATCAGATGAGGACACAGGCACACA-3'; *Ppp2r2d*, F: 5'-CGTG AACAAGAGAATAAAAGCCG-3', R: 5'-CTTCAATATTGGGA CCCGTAG-3'; *Ppp2r3a*, F: 5'-ACGCTTGTTGCAGAGGAATC-3', R: 5'-TCCAAATTCAGAGGGAGAGG-3'; *Ppp2r3c*, F: 5'-TC GTCGGCGCCTAGCGACGCCCAACACCTG-3', R: 5'-ATCG CTTCCTCTCCAATCATAGGTGGTGTCTGGTGTTTGTCCAGC-3'; *Ppp2r4*, F: 5'-GCTGAGGGCGAGCGGCAGCCGCCGCCA-3', R: 5'-GCCAGATGGGTAGGGACCACTGTGGCCACC-3'; *Ppp2r5a*, F: 5'-GAGTATGTTTCAACTAATCGTGGTGTAATTGTTGAATCA GCG-3', R: 5'-TCCCATAAATTCGGTGCAGAACAGTCTTCAGG-3'; *Ppp2r5b*, F: 5'-GACAACTGCCACACTGTGCT-3', R: 5'-TCC AGCTTGTAGGAGGCTGT-3'; *Ppp2r5c*, F: 5'-GTAATAAAGC GGGCAGCAGG-3', R: 5'-CAAAGT CAAAGAGGACGCAACA-3'; *Ppp2r5d*, F: 5'-AACTCCAAGAGCCACTGGAA-3', R: 5'-TGC CACATCTCTTCCCTTTC-3'; and *Ppp2r5e*, F: 5'-AAGCCAGACA GAAGAGGTCGCA-3', R: 5'-AGGAACAGTTCAGGCTGCTCTG-3'. Amplification was performed using the following conditions: denaturation at 95°C for 10 min, followed by 46 cycles of annealing at 60°C for 10 s and extension at 72°C for 10 s. Dissociation curve analyses confirmed that the signals corresponded to unique amplicons. Each experiment was performed in triplicate, and results were normalised against the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) obtained from parallel assays. Data were analysed using the LightCycler 2.0 System software package (Roche Diagnostics).

*Immunocytochemistry*. Standard protocols for immunofluorescence analysis were followed. Briefly, MC3T3-E1 cells were fixed with 3.7% formaldehyde and 0.2% glutaraldehyde, blocked with 5% skim milk in PBS, and incubated overnight with anti-RelA antibody (1:100) and anti-PR55β antibody at 4°C. The next day, the cells were incubated with Alexa Fluor 430-conjugated anti-rabbit IgG (1:10,000; Invitrogen, Carlsbad, CA, USA) for 90 min at 37°C. The
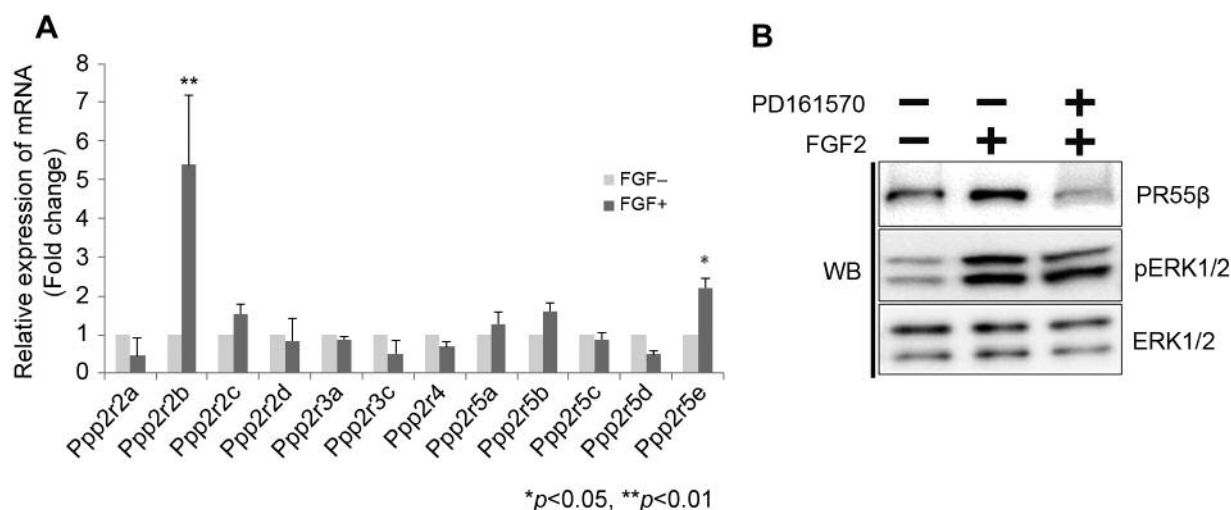
Figure 1. *FGF2 increases the expression of PR55β via ERK1/2 phosphorylation. (A) Relative mRNA levels of various regulatory B subunits in MC3T3-E1 cells as determined by qRT-PCR. Each bar represents the mean and standard error of five independent experiments. \*p<0.05, \*\*p<0.01. (B) Western blotting analysis of PR55β after separation of equal amounts of protein from total lysates of MC3T3-E1 cells using SDS-PAGE. FGF2 with or without PD 161570 (10 nM) was used for stimulation. Total ERK1/2 was used as a loading control.*

subcellular localisation was determined *via* fluorescence microscopy using a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan). To visualise the nuclei, cells were stained with 4',6-diamidino-2-phenylindole.

*Western blotting*. Cell lysates were homogenized using lysis buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 6% mercaptoethanol] containing protease inhibitor cocktail (Sigma-Aldrich). The protein content of the lysates and fractionated samples was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein from each sample containing 30 μg of protein were used for SDS-PAGE and western blotting with nitrocellulose membranes (Bio-Rad Laboratories). After washing with TBST [25 mM Tris-HCl (pH 8.2), 144 mM NaCl, and 0.1% Tween 20], the membranes were blocked with 5% skim milk in TBST at room temperature, and then incubated with specific antibodies. Immunoreactive bands were visualised using horseradish peroxidase-conjugated secondary antibodies (DAKO, Carpinteria, CA, USA) and ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The bands were quantified by computer-assisted densitometry (MultiImagerII Mi-II600CB; Bio-Tools, Maebashi, Japan).

*Nuclear extraction and NF-kB p65 activity assay*. MC3T3-E1 cells were collected in PBS/Phosphatase inhibitor solution, and the lysate was cleared by centrifugation at $300 \times g$ for 5 min. The lysate was incubated with 10% Nonient P-40 and centrifuged for 30 s at 4°C followed by resuspension of the pellet in nuclear extraction buffer containing 10 mM HEPES-NaOH (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol, and centrifugation at $14,000 \times g$ for 10 min at 4°C. The supernatant was used for NF-kB p65 activity assay, and the activity was measured by a kit obtained from Abcam (Cambridge, MA, USA).

*Immunoprecipitation*. MC3T3-E1 cells were collected in RIPA buffer containing protease inhibitors, followed by rotation at 4°C for 1 h, and the lysate was cleared by centrifugation at $15,000 \times g$ for 30 min. The lysate was incubated overnight with 2 μg anti-RelA rabbit polyclonal antibody at 4°C and then mixed with 10 mg of protein G-Sepharose beads followed by additional rotation at 4°C for 1 h. After rotation, the beads were washed thrice with lysis buffer, boiled in SDS-PAGE sample buffer for 5 min, and then subjected to western blotting.

*GST pull-down assays*. The purified protein of GST-TA2 was first incubated with glutathione Sepharose 4B (GE Healthcare, Canada) in a reaction buffer containing 20 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol for 30 min at 4°C. After washing with the same buffer, the beads were incubated with MC3T3-E1 cell lysates at 4°C for 30 min. At the end of incubation, the beads were washed and boiled in 50 μl sample buffer for 5 min, and subjected to SDS-PAGE and western blotting. Bound GST-TA2 and PR55β were detected using the antibodies.

*Statistics*. All statistical analyses were performed using GraphPad Prism (GraphPad Software).

## Results

*Effect of FGF2 on expression of the regulatory subunit of PP2A in MC3T3-E1 cells*. First, we examined the effect of FGF2 on the expression of the regulatory subunit of PP2A in MC3T3-E1 cells. *Ppp2r2b* coding for PR55β was clearly expressed and increased upon FGF2 stimulation (Figure 1A). To confirm whether the expression of PR55β depends on FGF signalling activation by FGF2 *via* FGFR, we examined
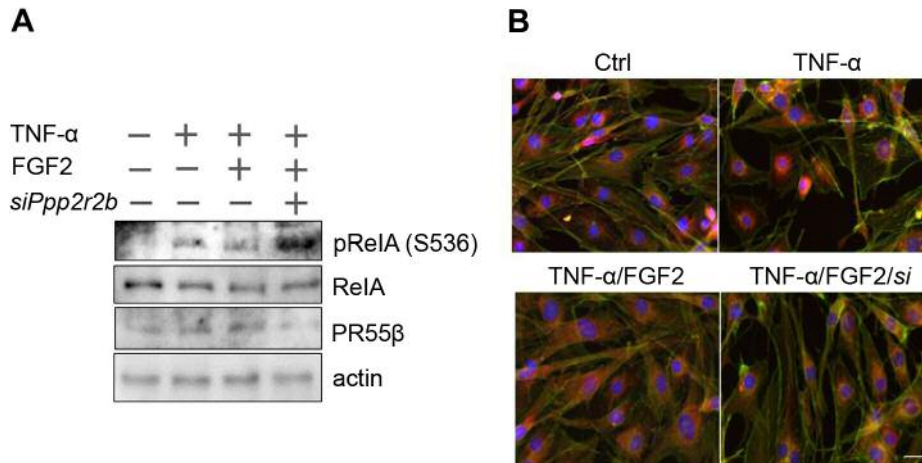
**A**

TNF-α    −   +   +   +
FGF2    −   −   +   +
*siPpp2r2b*   −   −   −   +

pRelA (S536)

RelA

PR55β

actin

**B**



Figure 2. *FGF2 regulates NF-kB signalling through dephosphorylation of RelA. (A) Dephosphorylation of RelA was detected by anti-phospho RelA antibody in western blotting analysis. (B) Immunocytochemistry analysis of total RelA in MC3T3-E1 cells transfected with siPpp2r2b or negative control siRNA with or without TNFα and FGF2, fixed with 3.7% formaldehyde and 0.2% glutaraldehyde, blocked with 5% skim milk in PBS, and incubated with the indicated antibody (1:250). Scale bar, 10 μm.*

the effect of PD161570, an inhibitor of FGFR, on FGF2 stimulation. Total ERK1/2 protein levels remained unchanged, however phosphorylated ERK1/2 levels were upregulated by FGF2 and the phosphorylation was suppressed by PD161570 (Figure 1B). Results showed that PR55β expression was promoted by FGF2 stimulation in MC3T3-E1 cells.

*Increased RelA dephosphorylation by FGF2 stimulation in MC3T3-E1 cells.* In MC3T3-E1 cells treated with TNFα, we confirmed that RelA is phosphorylated. We then examined the effect of FGF2 on the dephosphorylation of RelA. Interestingly, RelA was dephosphorylated by FGF2 stimulation. This effect was suppressed by the knockdown of PR55β using siRNA transfection (Figure 2A). Using immunocytochemistry (ICC), we also investigated whether nuclear NF-kB translocation including RelA was suppressed upon FGF2 stimulation. Results showed that while the translocation was increased by TNFα stimulation, it was suppressed by FGF2 stimulation, and surprisingly this effect was recovered by transfection with siRNA against PR55β (Figure 2B). These data suggest that activation of FGF signalling by exogenous FGF2 suppresses NF-kB signalling through the dephosphorylation of RelA and expression of PR55β.

*Effect of PR55β on NF-kB activity.* Next, we examined NF-kB activity after stimulation by TNFα. Because RelA phosphorylation is related to its activity *via* translocation to the nucleus, which is essential for signal activation, the activity of NF-kB was up-regulated by TNFα stimulation,
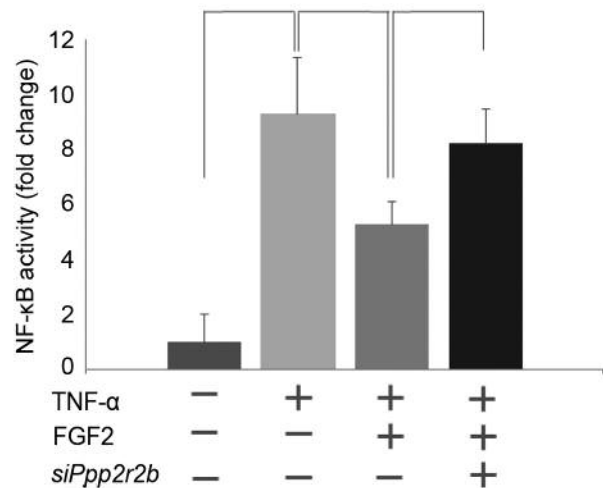


Figure 3. *PR55β is related to NF-kB signalling activity. NF-kB signalling activity was measured using a prepared kit. FGF2 stimulation decreased this activity through the expression of PR55β.*

but was suppressed by FGF2 stimulation; the effect was recovered by transfection with siRNA against PR55β (Figure 3). These data suggested that PR55β is involved in not only the phosphorylation of NF-kB, but also its activity.

*Binding of RelA to PR55β.* We confirmed the binding between RelA and PR55β using immunoprecipation assay and western blotting using specific antibodies. RelA was co-immunoprecipitated with PR55β compared to the negative
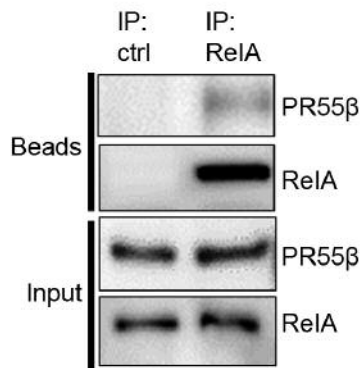
Figure 4. *PR55β binds to RelA in response to FGF2 stimulation. Immunoprecipitation assay was performed using an anti-RelA antibody immobilised by protein G-Sepharose beads.*



Figure 5. *PR55β interacts with RelA by binding to the TA2 domain of RelA. (A) Domain structure of RelA. RHD is the Rel homology domain, and TA1 and TA2 domains are transactivation domains. (B) The recombinant proteins GST-TA2 or GST alone were purified, and their size and concentration were confirmed by Coomassie brilliant blue (CBB) staining. (C) Binding between PR55β and GST-TA2 was investigated by GST-pull-down assay.*

control antibodies (Figure 4). Input lysates were sufficient for binding. These results additionally suggest that signal transduction *via* PR55β, one of the regulatory subunits of PP2A, is important for this interaction.

*Direct interaction between the TA2 domain of RelA and PP2A via PR55β.* Finally, we investigated the direct interaction between the TA2 domain of RelA and PP2A *via* PR55β. The domain structure of RelA is shown in Figure 5A. In addition to the Rel homology domain (RHD) of the N-terminal side, transactivation domains 1 (TA1) and 2 (TA2) are located between amino acids 428 and 550. To investigate the interaction between RelA and PR55β, we constructed the recombinant protein of glutathione-S-transferase (GST)-tagged TA2 domain and GST alone, and confirmed their expression and size by Coomassie staining (Figure 5B). Finally, in a GST-tag pull-down assay using the recombinant protein and cell lysate, we detected the PR55β immobilised to the TA2 domain of RelA by western blotting (Figure 5C). These data showed direct interaction between the TA2 domain of RelA and PP2A *via* PR55β.

*Schematic representation of the regulatory mechanisms of FGF2 via PR55β expression during RelA inactivation.* FGF2 stimulation promotes PR55β expression through ERK phosphorylation. On the contrary, upon activation of the NF-kB signalling pathway, activated RelA is dephosphorylated by the interaction of PR55β with the TA2 domain of RelA (Figure 6).

## Discussion

FGF signals are implicated in the suppression of inflammation. Inflammation is caused by cell stimulation from a trauma or an infectious disease, which further
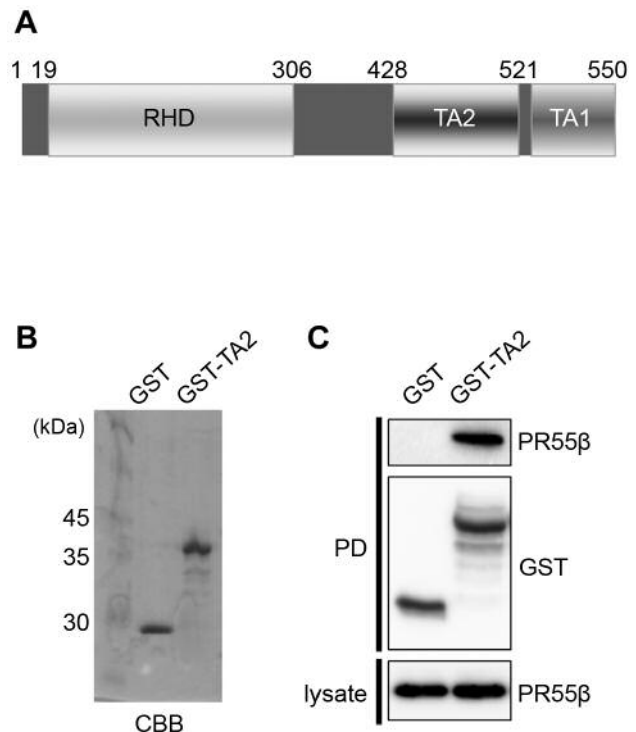
accelerates inflammation and releases inflammatory cytokines. Activation of NF-kB signalling results in severe bone impairment and expands around tissues through the phosphorylation and translocation of RelA. A recent study suggested the possibility that regulation by FGF signals contributes to the dephosphorylation of NF-kB targeting molecules such as IKK or I-kB, as well as RelA (16). Therefore, we focused on the involvement of RelA in the regulation of NF-kB signalling and FGF2-related expression of phosphatases and elucidated the mechanisms underlying this crosstalk.

Although PP2A is ubiquitously expressed and is known to be necessary for regulating the inflammatory response, expression of the regulatory subunits of PP2A under FGF2 stimulation remains unclear. Therefore, in this report, we investigated the expression of many genes encoding the regulatory molecules of the PP2A family members by performing quantitative RT-PCR and western blotting analysis, and we found that PR55β is expressed in pre-osteoblastic MC3T3-E1 cells (Figure 1). During embryonic
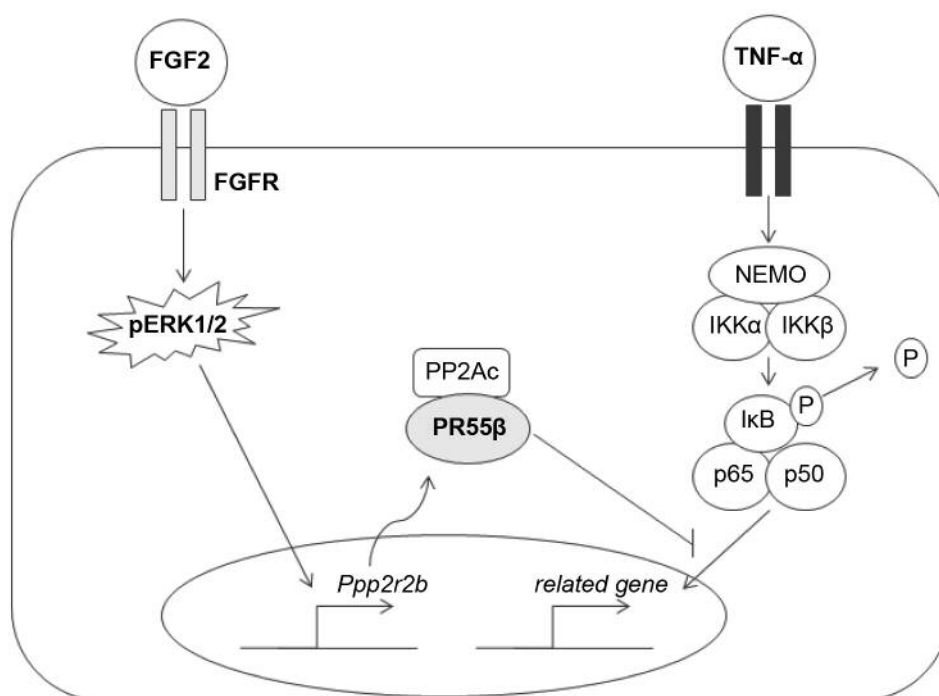
Figure 6. *Schematic representation of the proposed mechanism.*

development, PR55β is expressed at the foetal stage, and its expression decreases over the course of development. It is expressed mainly in the brain and testes and at low levels in the lungs and spleen (17). Increased expression of PR55β has been observed in embryos during neuronal differentiation, suggesting that it is essential for embryonic development (18). Its expression was increased upon FGF2 stimulation, suggesting that PR55β is a target molecule for FGF2-related signals. PR55β might have functions similar to those observed in the development or differentiation in osteoblasts. Moreover, a recent study revealed that PR55β is expressed in several cancers (19). Indeed, PR55β expression has been found to be associated with colorectal cancer (20). Cell lines originating from human adenoid cystic carcinoma (AdCC) showed a relationship between PR55β expression and the growth and metastasis of AdCC (21). We confirmed that increased cell proliferation was suppressed upon PR55β knockdown (data not shown). Given that PR55β is expressed in response to cell growth or proliferation, the role of PR55β might be to accelerate the progression of cell proliferation.

Activation of FGF signalling by exogenous FGF2 may regulate NF-kB because of the suppressive effect of FGF2 on inflammation (22). Thus, we investigated the relationship between phosphorylated RelA and FGF2 stimulation. Notably, the phosphorylation of RelA activated by TNFα was decreased upon FGF2 stimulation, suggesting that PR55β

regulates their crosstalk (Figure 2). RelA phosphorylation is critical for the activation of NF-kB signalling, and translocated RelA binds to DNA and promotes the expression of related genes (23). Signal activation responsible for the dephosphorylation of RelA is implicated in the regulation by FGF2. We thus investigated whether NF-kB activity is suppressed upon FGF2 stimulation by measuring the ability of RelA to bind to DNA. The effect of decreasing PR55β was observed not only on the phosphorylation level of RelA, but also on NF-kB activity (Figure 3). These results led us to hypothesise that PR55β interacts with RelA. In an immunoprecipitation assay, PR55β was bound to RelA through direct interaction (Figure 4).

Direct binding to the target substrate is required for proper regulation by PP2A complexed with PR55β. RelA has two distinct domains, TA1 and TA2, which are necessary for its nuclear translocation and activation (24). The TA1 domain includes the phosphorylation site Ser539, which is responsible for RelA activation, whereas the TA2 domain serves as a target for many interacting molecules including kinases and phosphatases (25). Therefore, to determine whether the interaction involves direct binding *via* the TA2 domain, we constructed and purified GST-tagged recombinant proteins, GST, or GST-TA2. First, we confirmed their expression and size by Coomassie staining (Figure 5B). Finally, in a GST-tag pull-down assay, we detected the

PR55β protein by western blotting (Figure 5C). The phosphorylation site, Ser536, of the TA1 domain is critical for RelA activation and translocation, which provides new insights into the binding site of RelA with PP2A. Taken together, these data suggest that PR55β is recruited to RelA by FGF2 stimulation and supports the dephosphorylation of RelA by PP2Ac.

In summary, we demonstrated the importance of the regulatory mechanism of RelA dephosphorylation through interaction between PR55β and NF-kB signalling. Specifically, we demonstrated an efficient mechanism by which signalling crosstalk occurs between FGF2 and NF-kB. However, additional studies are required to confirm the *in vitro* experiments using recombinant proteins *in vivo*.

## Conflicts of Interest

The Authors have no potential conflicts of interest to disclose.

## Authors' Contributions

A.S. and G.S. carried out the experiments. G.S. wrote the manuscript and with support from W.K. and T.Y. helped supervise the project. Y.O. conceived the original idea. Y.M. supervised the project.

## Acknowledgements

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