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# Regulated Expression of Syndecan-4 in Rat Calvaria Osteoblasts Induced by Fibroblast Growth Factor-2

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**Abstract** Fibroblast growth factor-2 (FGF2) is a member of a prominent growth factor family that drives proliferation in a wide variety of cell types, including osteoblasts. The binding and signal transduction triggered by these mitogens is dependent on glycosaminoglycan (GAG) sugars, particularly of the heparan sulfate (HS) class. These are secreted in proteoglycan (PG) complexes, some of which become FGF co-receptors. The syndecans, the transmembrane forms of HSPG of which there are four members, act as multifunctional receptors for a variety of ligands involved in cell-extracellular matrix (ECM) adhesion as well as growth factor binding. To understand the role of syndecans in developing osteoblasts the effects of exogenous FGF2 on syndecan expression were examined using primary rat calvarial osteoblasts. All four syndecan mRNAs were expressed in the osteoblasts, although only syndecan-4 was upregulated by FGF2 treatment in a dose-dependent manner. This upregulation could be abrogated by pretreatment with the protein synthesis inhibitor cycloheximide, suggesting that the upregulation of syndecan-4 by FGF2 is not a primary response. Osteoblast proliferation and mineralization were enhanced by exogenous FGF2 treatment, but could be specifically diminished by anti-syndecan-4 antibody pretreatment. This treatment also blocked FGF2-induced extracellular signal-regulated kinase activation, but not the expression of the bone-specific transcription factor Runx2. These results demonstrate that mitogen-triggered syndecan-4 expression is an intrinsic part of the pathways subtending osteoblast proliferation and mineralization. J. Cell. Biochem. 100: 402–411, 2007. 

**Key words:** proteoglycan; bone; mitogen; heparan sulfate

Fibroblast growth factor-2 (FGF2) drives the growth, migration, and differentiation of a wide variety cells types [Ornitz and Itoh, 2001] including the precursor cells of developing bone. It does this by stimulating osteoblast proliferation [Shimoaka et al., 2002], differentiation [Marie, 2003; Fakhry et al., 2005], and bone sialoprotein (BSP) gene expression, thereby inducing cell mineralization [Chaudhary et al., 2004]. FGF functions cells through bringing to their high-affinity receptors which are expressed by rat calvarial osteoblast cells [Song et al., 2005]. The interaction of FGF2 and their receptors is controlled by a low-affinity, high-capacity interaction with the heparan sulfate (HS) side-chains of proteoglycans (PGs). These molecules act as co-receptors for a plethora of heparin-binding growth factors [Bernfield et al., 1999], and the activation of high-affinity cell surface, cognate FGFRs is known to be dependent on the presence of cell surface heparin-like molecules [Bansal and Pfeiffer, 1994; Rapraeger et al., 1994].

There are many forms of HSPG, including those resident within the extracellular matrix (ECM) and the pericellular space. There is a family of transmembrane HSPGs, known as syndecans, which have four members (syndecans-1 to -4) in mammals, and which have been implicated in a number of important functions, including mitogen binding [Aviezer et al., 1994],

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cell adhesion [Salmivirta et al., 1991], and homeostasis [Kojima et al., 1992]. Overexpression of syndecan-1 inhibits the FGF2-stimulated proliferation of some cells [Mali et al., 1993], whereas syndecan-4 is known to bind heparin-binding epidermal growth factor (HB-EGF), which is a component of wound fluid that stimulates smooth muscle-cell migration [Higashiyama et al., 1993]. Syndecans-1, -2, and -4 are co-expressed in rat osteoblasts [Molteni et al., 1999b], and appear to promote the proliferation and differentiation that occurs during both bone development and fracture healing. The purported functional role of these HSPGs in osteogenesis is supported by a finding that alterations in matrix- and cell-associated PG expression inhibit osteogenesis and growth response to FGF2 in cultured rat mandibular condyle and calvaria cells [Molteni et al., 1999a]. As well as this, syndecans-2, -4 appear to play roles in cartilage differentiation [Molteni et al., 1999b].

Although it is the HS side-chains of HSPGs that are thought to play the major bioactivating roles during FGF2-signaling, some studies have suggested that syndecan core proteins may also play an important role in signal transduction. Syndecan-4, the most widely expressed member of the family, differs in its sequence from the other three syndecans by a unique phosphatidylinositol 4,5-bisphosphate-binding site located in its cytoplasmic tail [Lee et al., 1998; Horowitz et al., 1999] that is thought to become active during FGF2 signal transduction [Volk et al., 1999; Horowitz et al., 2002]. Indeed, syndecan-4 has been suggested to be a bona fide signaling receptor because of its ability to bind and activate protein kinase-Cα (PKCα) upon FGF2-induced oligomerization [Oh et al., 1997; Simons and Horowitz, 2001]; such PKCα activation has been linked to the promotion of cell growth [Oh et al., 1997; Lallena et al., 1999; Besson and Yong, 2000]. However, the regulation of syndecans by FGF2 in rat calvarial osteoblasts has not been reported.

The purpose of this study was to examine the expression of syndecan genes in cultured primary rat osteoblasts and to investigate the regulation effects of FGF2 on syndecan expression. The functional consequences of changes in syndecan-4 composition of the rat osteoblast in responses to FGF2 were also studied.

## MATERIALS AND METHODS

### Isolation and Culture of Primary Osteoblasts

Primary rat calvarial bone cell cultures were generated by sequential collagenase digestion of calvariae removed from neonatal rats within 24 h of birth as described [Asahina et al., 1993]. These were cultured to confluence, then trypsinized, pooled, and replated at 5,000 cells/cm$^2$ for further experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin G-sodium, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin, and 2 mM L-glutamine at 37°C under 5% $CO_2$ in air. To induce differentiation, cells were grown in an osteogenic medium which consisted of DMEM supplemented with dexamethasone (Sigma, St. Louis, MO; 10 nM), β-glycerophosphate (Sigma; 10 mM), and ascorbic-2-phosphate (Sigma; 50 μM). Cell culture medium and additives were from Invitrogen (Carlsbad).

### Proliferation Assay

Cell proliferation was assayed by 5-bromo-2′-deoxyuridine (BrdU) incorporation (Roche, Nutley, NJ). Primary osteoblasts were cultured in 96-well plates until sub-confluent. Cells were washed and deprived of serum for 24 h, then treated with different substances for 72 h, and labeled with BrdU for the last 24 h. Cells were fixed, and incorporated BrdU detected by a direct immunoperoxidase method according to the manufacturer's instructions. The absorbance was read at 450/630 nm in a Wallac Victor3 1420 Multilabel Counter (Perkin Elmer, Wellesley).

### Mineralization Assay

Primary osteoblasts were seeded into 24-well plates and allowed to settle overnight, and then exposed to normal rabbit serum with or without an anti-syndecan-4 antibody for 2 h. Cells were treated with increasing concentrations of FGF2 in osteogenic medium for an initial 3 days, and then grown continuously in osteogenic medium for further 14 days. Cells were fixed with 4% paraformaldehyde (Sigma) in PBS for 30 min. Mineral deposition was detected with Von Kossa Stain. Cultures were incubated with 1% silver nitrate (Sigma) for 20 min under UV light and washed in three changes of distilled water. The cultures were treated with 5% sodium thiosulphate (Sigma) for 2 min, washed in

distilled water for three times. Nodules were then counted by eye.

### RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was extracted from cell samples using a NucleoSpin RNA II Kit according to the manufacturer's instructions (Macherey-Nagel, Easton, PA). Polymerase chain reaction was used to investigate the expression of syndecans. The first strand cDNA synthesis was carried out on total RNA using SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen). Primers for the rat PGs, ALP, Runx2, and hypoxanthine-phosphoribosyltransferase (HPRT) were synthesized by Proligo (Proligo LLC, Boulder, CO). Sequences are listed in Table I. Quantitative PCR was then performed to assess the relative expression of the syndecans in rat osteoblasts. This was carried out on an ABI Prism 7000 Sequence System (AB Applied Biosystems, Warrington, UK) using 250 nmol/L forward and reverse primers and SYBR Green PCR Master Mix (AB Applied Biosystems). Reactions were run on the following thermal profile; hot start at 95°C for 10 min, then denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, all for 40 cycles. Data were collected to the SYBR channels at the end of each extension step, and the final cycle was followed by a thermal melt step. Data collected for each sample were then analyzed using the $2^{\Delta\Delta C_T}$ method [Livak and Schmittgen, 2001] to give a relative expression (normalized to HPRT expression in each sample).

### Western Blotting

Primary osteoblasts were cultured in six-well plates until sub-confluent. Cells were washed and deprived of serum for 24 h and then treated with different agents for 30 min for extracellular signal-regulated kinase (ERK1/2) assessment. Cells were lysed in ice-cold buffer (150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 0.1% SDS, Tris 10 mM (pH 7.4), Triton X-100 1%) containing a protease inhibitor cocktail (Sigma). Aliquots of cell lysate containing 6 μg of total protein were separated on 10% (w/v) SDS–polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking with 5% nonfat milk in buffer (1.5 M NaCl, 1 M Tris, pH 7.4, 1% Tween) for 1 h, proteins were incubated with primary antibodies (anti-pERK1/2, Sigma) overnight at 4°C followed by an incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody. Specific bands were detected on X-ray films using the ECL chemiluminescence detection method (Pierce, Rockford). Membranes probed with pERK were stripped and reprobed with anti-ERK1/2 (Sigma) and then with anti-actin (Chemicon, Temecula).

### Immunocytochemistry

Cells were grown in four-chamber slides until sub-confluent, then washed, deprived of serum for 24 h, and then treated with the nominated substances for 24 or 48 h in serum-free medium. Cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS, then permeabilized with 0.3% Triton X-100 in blocking buffer (5% normal goat serum in PBS) for 30 min. Cells were incubated with 6.25 μg/ml (1:40) rabbit anti-syndecan-4 primary antibodies or the same concentration of normal rabbit serum (as negative control) overnight at 4°C, washed three times with PBS, and then incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen; 1:500 in blocking buffer) for 2 h. Cells were

**TABLE I. Primers Used in Quantitative RT-PCR**

| Name of genes | Acc. no | Sequences of forward primers (5′–3′) | Sequences of reverse primers (5′–3′) | Size |
|---|---|---|---|---|
| *HPRT* | NM_012583 | TTTGCTGACCTGCTGGATTACA | TTATGTCCCCCGTTGACTGGT | 121 |
| *Syndecan-1* | NM_013026 | CCTTCATCCCTCATGGTTGCT | AGATCTTTTGTCTAGCCGCGC | 112 |
| *Syndecan-2* | BC070890.1 | ACTGTCAAAGCTCCCTTGCAG | TGTCGTTTTAACATGCAGAGCC | 101 |
| *Syndecan-3* | NM_053893.2 | ACGTTGCTCATCTACCGCATG | TAGAACTCCTCCTGCTTGTCGG | 110 |
| *Syndecan-4* | NM_012649 | TGGCCACACAATTTAAGTGCC | TCCTAAGTCCCCTTCTCTTGCC | 116 |
| *Perlecan* | U75305.1 | CCTGAAGTACCTGAGACCATCG | CCATCCTGTAGTCCAAGGCTAA | 128 |
| *Glypican-1* | NM_030828 | TCCCGACTATTGCCGAAATGT | GTCAGTGATGAGCACCATGGAG | 103 |
| *Glypican-3* | NM_012774.1 | CCCTGTGCCAGGATCAGATTT | ATGTTCAGGCGCGCTGTTAGT | 102 |
| *ALP* | NM_013059.1 | CGAGCAGGAACAGAAGTTTGC | TGGCCAAAAGGCAGTGAATAG | 105 |
| *Runx2* | AF053953.1 | TCTGAGCACAGTCCATGCAGT | TGTTTGACGCCATAGTCCCTC | 104 |
| *Biglycan* | BC072480.1 | CAGGAACATTGACCATGCGTC | GCCCATCATCCAAGGTGAAGT | 111 |
| *Decorin* | XM_343201.1 | ACCCGGATTAAAAGGTGGTGA | TCTCTGCTCAAATGGTCCAGC | 104 |
| *Versican* | AF072892.1 | TTTGCTCATCGCCGTACATG | GCCCACACGATTCACAAACA | 111 |

washed with PBS, mounted, and examined under confocal microscopy.

### Total DNA Quantification

Cultures were seeded in 96-well plates and cells allowed to settle overnight. Cells were incubated in normal rabbit serum with syndecan-4 antibodies for 2 h and then treated with different concentration of FGF2 in osteogenic medium for a further 3 days. The DNA content of cultures was determined with a PicoGreen dsDNA quantification kit (Molecular Probes, OR) according to the manufacturer's instruction. Briefly, cultures were washed with PBS, whereupon distilled water (100 μl) was added to each well and cells frozen and thawed four times; TE buffer (100 μl) was then added to each well. Cells were centrifuged, and two aliquots of supernatant (50 μl) were transferred an ELISA plate. PicoGreen dye (50 μl) was added to each well, incubated for 5 min in darkness, and the absorbance read at 480 nm in a Wallac Victor3 1420 Multilabel Counter. The amount of DNA was then calculated from a standard curve.

### Statistics Analysis

All results were analyzed using two-tailed unpaired Student's *t*-test assuming unequal variances; $P$-values $< 0.05$ were considered significant.

## RESULTS

### Expression of Syndecans in Rat Primary Osteoblasts Is Regulated by FGF2

To determine the expression of PGs in rat osteoblasts and their possible modulation of syndecan expression by FGF2, quantitative RT-PCR was performed on RNA derived from rat calvarial primary osteoblasts (Fig. 1A). Biglycan is the most abundant PG in these cells, followed by the HSPGs glypican-1 and perlecan; syndecans were expressed at comparatively lower levels as compared to other PGs. Of the four syndecans, syndecan-2 mRNA is the most abundant, followed by syndecans-4, -1, and -3 (Fig. 1B). In order to examine whether FGF2 could specifically modulate this expression pattern, quiescent rat primary calvarial osteoblasts were exposed to different concentrations of this mitogen in serum-free medium for 6 h. FGF2 specifically upregulated syndecan-4 mRNA expression levels for these cells in a dose-dependent manner (Fig. 1C). This increase was twofold at 1 ng/ml of FGF2, and threefold at 10 ng/ml, with the other syndecans remaining unchanged. To determine whether the increase in mRNA resulted in an increase in actual syndecan-4 PG, cells were treated with or without FGF2. Immunocytochemistry (Fig. 1E–G) was then performed with results showing a stronger staining for syndecan-4 in cells treated with FGF2 than control cells. Syndecan-4 was predominantly localized to the cell membrane (Fig. 1E,F).

### Enhancement of Syndecan-4 Gene Expression by FGF2 Is an Indirect Effect

The products of primary-response genes are thought to be responsible for the functional changes required to initiate cell division [Herschman, 1991; Simons et al., 1992]. To investigate whether *syndecan-4* gene upregulation in reaction to increased FGF2 levels is a primary response, the protein synthesis inhibitor cycloheximide was used. Quiescent rat osteoblasts were treated with this agent (100 μg/ml) for 2 h and the cells then exposed to exogenous FGF2 (10 ng/ml) for 6 h. Quantitative RT-PCR was then performed on RNA derived from these cultures. Syndecan-4 mRNA expression was dramatically reduced by the of cycloheximide pretreatment (Fig. 1D), indicating that the induction of the *syndecan-4* gene is not an immediate response, but rather requires new protein synthesis.

### Syndecan-4 Blockade Abrogates FGF2-Induced Proliferation

To assess whether the enhanced syndecan-4 expression seen after FGF2 addition in the rat osteoblasts is correlated with any particular function, quiescent cells were first treated with different concentrations of FGF2 and their proliferation assessed by BrdU incorporation. FGF2 induced a significant increase in BrdU incorporation at lower (0.1–1 ng/ml), but not higher concentrations (10 ng/ml; Fig. 2A). However, if cells were incubated with syndecan-4 antibodies before exposure to FGF2, the anti-syndecan-4 antibodies could block the effects of FGF2 on cell proliferation (Fig. 2A); this blockade was also evident at the level of MAPK activation (Fig. 2B).

### FGF2 Influences Progenitor Cell Differentiation and Mineralization

Nodule formation was next used to assess the differentiative effect of FGF2 on osteoblast cell mineralization. FGF2 stimulated this process in

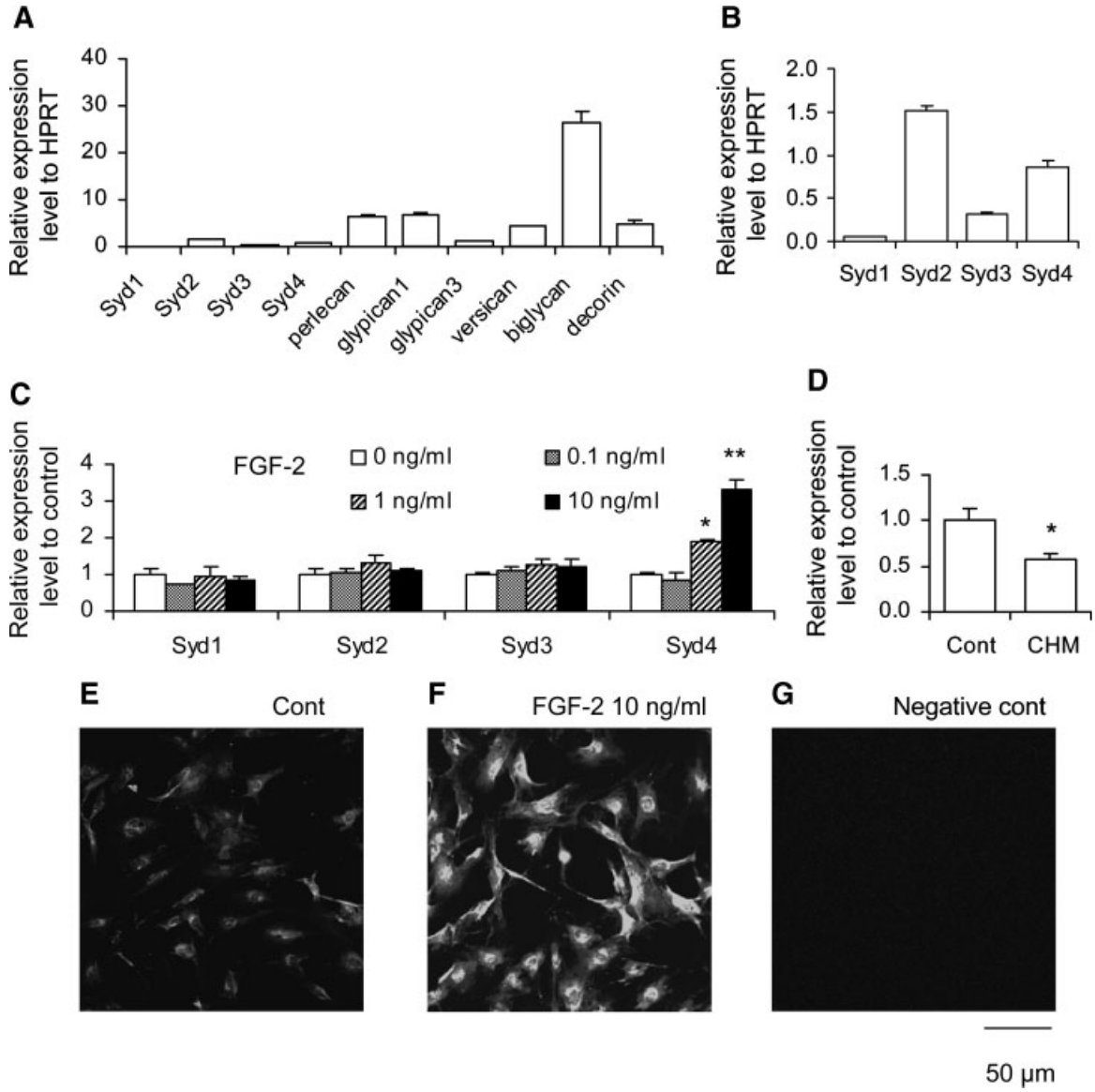


**Fig. 1.** The expression of proteoglycan core protein in rat cavarial osteoblasts and the regulation of syndecan expression by FGF-2. **A**: Relative mRNA expression levels of proteoglycan to HPRT in rat calvarial osteoblasts evaluated by quantitative RT-PCR. **B**: Enlargement of relative syndecan (Syd) mRNA expression levels in A. **C**: The regulation of syndecan mRNA expression by FGF-2. Cells were grown to sub-confluence, deprived of serum for 24 h and then exposed to FGF-2 in serum-free medium. RNA was analysed by quantitative RT-PCR for the relative expression of syndecans. **D**: Cells were grown to subconfluence, deprived of serum for 22 h and pretreated with ethanol vehicle (Cont) or 100 ug/ml cycloheximide (CHM) in serum-free medium for 2 h, and then all cells were treated with 10 ng/ml FGF-2 for 6 h. RNA was extracted from these cells and syndecan-4 expression assessed with quantitative RT-PCR. Data are presented as a relative expression level over relevant control values (mean ± SEM; n = 4). Significant differences between values for different treated and relevant control values are indicated as *($P < 0.05$) or **($P < 0.01$). **E**, **F** and **G**: Cells were grown to near confluence, deprived of serum and treated for 24 h without (E) or with 10 ng/ml FGF-2 (F and G). Cells were fixed and incubated with syndecan-4 antibody (E and F) or normal rabbit serum (G). Specific staining was detected by Alexa Fluor 488-labeled secondary antibody and photographed with fluorescence microscopy connected with laser scanning system.

a dose-dependent manner, with the increase in nodule formation reaching sevenfold at 10 ng/ml of FGF2 compared to control cells (Fig. 3A,B). The addition of anti-syndecan-4 antibodies was next used to assess whether this particular HSPG was involved in nodule formation. Cell number was determined by DNA quantification; as well as this, the expression of the bone-specific transcription factor Runx2, and the bone marker alkaline phosphatase (ALP) were

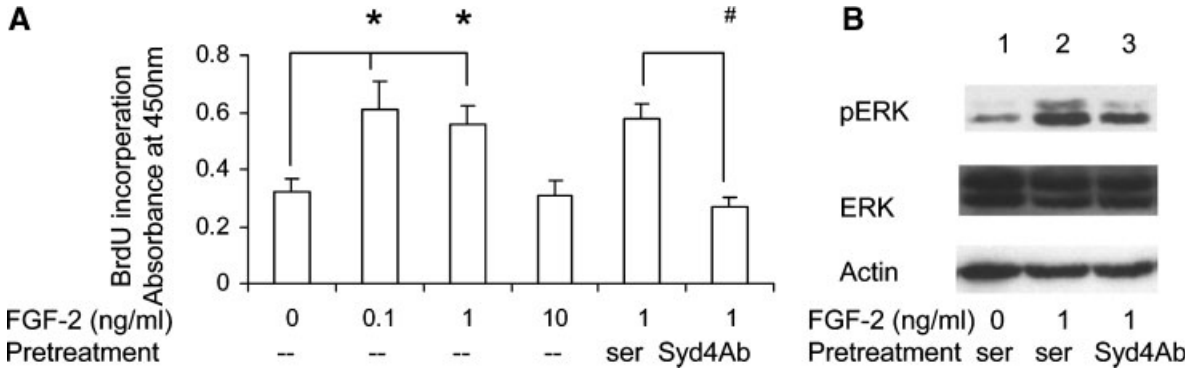


**Fig. 2.** The effects of blocking syndecan-4 on FGF2-induced rat osteoblast proliferation. **A**: Cells were grown to near confluence, deprived of serum for 22 h, pretreated with rabbit serum (ser; as control for syndecan-4 antibody) or with syndecan-4 antibody (Syd4Ab) for 2 h and then treated for 72 h with the indicated FGF-2 concentrations. Cells were labeled with BrdU for last 24 h. The data represent mean ± SEM (n = 5). Significant differences between values for FGF-2-treated and relevant control values are indicated as *($P < 0.05$), or between values for syndecan-4 antibody pretreated and relevant control values are indicated as #($P < 0.05$). **B**: Cells were grown to near confluence, deprived of serum for 22 h, pretreated with rabbit serum or with syndecan-4 antibody in serum-free medium for 2 h and then cells treated for 30 min with 1 ng/ml FGF-2. Total protein was extracted for Western blotting and first probed for actived ERK expression. Membranes were then stripped and re-probed with anti-ERK antibody and then anti-actin antibody.

also investigated with quantitative PCR under osteogenic conditions. It was found that FGF2 could induce cell proliferation in a dose-dependent manner even after 3 days in osteogenic conditions (Fig. 4A), as well as an increase in Runx2 mRNA expression (Fig. 4B). However, ALP expression was not affected. Syndecan-4 antibody treatment under osteogenic culture conditions did not significantly affect either the cell proliferation rate or the osteoblast marker or transcription factor gene expression in osteogenic condition (Fig. 4).

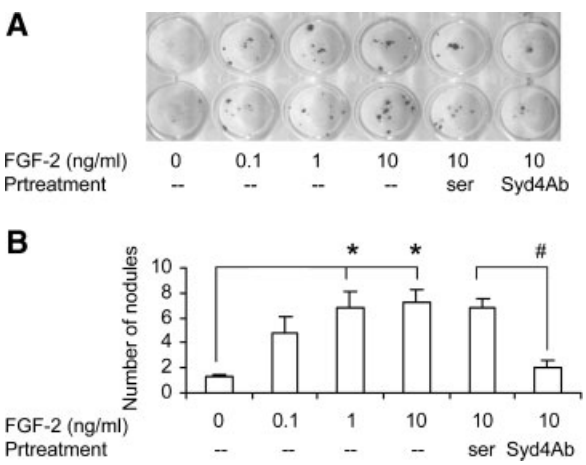


**Fig. 3.** The effect of syndecan-4 blocking during FGF2-induced rat osteoblast differentiation. **A**: Passaged cells were allowed to settle for 22 h, pretreated with rabbit serum (ser; as control) or syndecan-4 antibody (Syd4Ab) for 2 h, then treated with FGF2 in osteogenic culture medium for three days. Cells were then changed into osteogenic culture medium for further 14 days, fixed with 4% PFA and then Von Kossa stained. **B**: The number of nodules was counted manually. The data are presented as mean ± SEM (n = 4). Significant differences between values for FGF-2-treated and the relevant control values are indicated as *($P < 0.05$) and the values for syndecan-4 antibody-pretreated and relevant control values indicated as #($P < 0.05$).

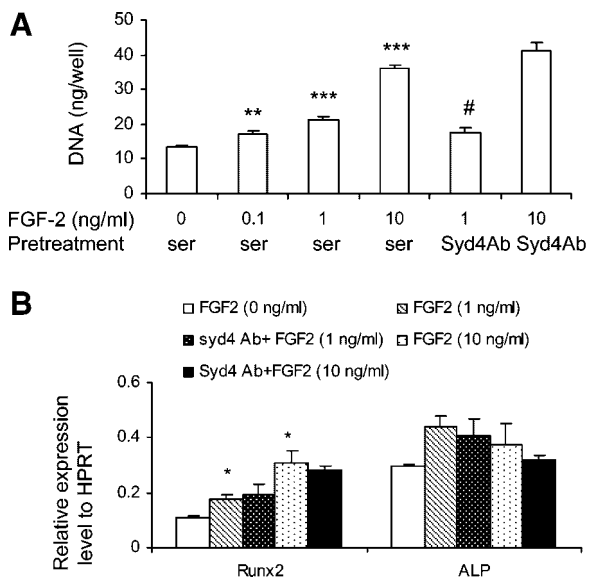


**Fig. 4.** The effect of FGF2 on osteoblast gene expression in osteogenic medium. Passaged cells were allowed to settle for 22 h, pretreated with either rabbit serum (ser; as control for syndecan-4 antibody) or syndecan-4 antibody (Syd4ASb) for 2 h, then treated with FGF-2 in osteogenic culture medium for three days. Cells were subjected to DNA quantification using the PicoGreen method (n = 5; **A**) and quantitative PCR analysis (n = 4; **B**). PCR data are presented as relative to the expression level of HPRT. Significant differences between values for FGF2-treated and the relevant control values are indicated as *($P < 0.05$); **($P < 0.01$) and ***($P < 0.001$) or between values for syndecan-4 antibody pretreated and relevant control values indicated as #($P < 0.05$).

## DISCUSSION

Heparan sulfate proteoglycans (HSPGs) are a unique family of macromolecules that consist of a core protein and one or more covalently attached glycosaminoglycan (GAG) chains found within the ECM, basement membranes, as well as on cell surfaces [Selleck, 2000] and which serve critical roles in growth and differentiation [Prydz and Dalen, 2000]. PGs, and particularly HSPGs, are known to mediate the cellular responses of many mitogens and adhesive factors; this study showed that a large number of PGs were expressed by rat calvarial osteoblasts, including the syndecan HSPGs. In response to FGF2, a major mitogenic influence on osteoprogenitors [Fakhry et al., 2005], syndecan-4 was specifically upregulated, although this was not a primary response. To test whether the syndecan-4 response was coupled to FGF2, blocking antibodies to the PG were employed. The results show that the responses of osteoprogenitors to FGF2 are in part coupled to syndecan-4 metabolism and availability.

Virtually all adhesive cells express at least one syndecan, and most express multiple syndecans [Kim et al., 1994]; interestingly, syndecan transmembrane core proteins are capable of carrying both HS and chondroitin sulfate chains. These latter are abundantly expressed by maturing bone cells [Slater et al., 1994]. Previous studies have shown that a rat osteoblast cell line (ROS 17/2.8) and human osteoblasts [Modrowski et al., 2000], all express syndecans-1, -2, and -4, and the results here confirm they are also expressed in rat primary osteoblasts. Indeed, rat osteoblasts express the mRNAs for all four syndecans.

In this study, FGF2 stimulated the expression syndecan-4, but not other syndecans, in rat osteoblasts, suggesting that different growth factors may use different syndecans for distinct cell functions. Growth factors are known to play an important role in the regulation of syndecan expression; for example, syndecan-1 mRNA is upregulated by PDGF but not by FGF2, and syndecan-4 expression can be induced by FGF2 in vascular smooth muscle cells [Cizmeci-Smith et al., 1993, 1997]. Syndecan-1 expression is enhanced by both FGF2 and TGF-β in 3T3 cells [Elenius et al., 1992; Jaakkola et al., 1997] and syndecan-3 is upregulated by FGF2 in oligodendrocytes [Winkler et al., 2002]. In addition to growth factors, syndecan expression can also be regulated by the complicated conditions of wound healing. Syndecans-1 and -4 are increased in arterial smooth muscle cells after balloon catheter injury [Nikkari et al., 1994], in skin wound healing [Elenius et al., 1991; Gallo et al., 1994], and in the heart following myocardial infarction [Li et al., 1997]. Syndecans are multifunctional regulators of cell morphology, growth, and differentiation, in part through their interactions with heparin-binding growth factors [Salmivirta et al., 1991; Kojima et al., 1992; Aviezer et al., 1994]. During tissue repair, cells are required to replace the damaged tissue through coordinated processes such as migration, proliferation, and differentiation, suggesting that syndecans might be central to these processes. Syndecan-4 induction by FGF2 was significantly diminished in the presence of cycloheximide, allowing it to be classified as secondary-response gene product, requiring the synthesis of new proteins. Further studies will be required to determine which signaling pathways are involved in FGF2-stimulated syndecan-4 expression.

Fibroblast growth factor-2 is most extensively studied and prototypical of the heparin-binding growth factors. Accumulating evidence has demonstrated that it plays important roles in regulating bone cell function as well as bone formation. It can significantly stimulate the proliferation of mouse primary osteoblasts [Shimoaka et al., 2002] as well as their differentiation [Marie, 2003; Fakhry et al., 2005]. FGF2 stimulates osteoprogenitor BSP gene expression during the process of cell mineralization [Chaudhary et al., 2004]. Abrogation of FGF2 gene expression results in decreased bone formation with coincident loss of bone mass [Montero et al., 2000]; provision of exogenous FGF2 promotes bone healing in vivo [Kawaguchi et al., 2001; Chen et al., 2004]. The major finding of this study was that the ERK activation, and thus the proliferation and enhanced cell mineralization, seen in FGF2-driven rat osteoblasts can be reduced by treatment with anti-syndecan-4 antibody. This suggests that syndecan-4 might be acting as the chief co-receptor for FGF2-signaling in these cells. Presumably the FGF2-induced syndecan-4 expression results in a greater proportion of the HS-carrying FGF-specific binding sites, resulting in the presentation of more FGF2 to cells surface cognate receptors [Klagsbrun and

Baird, 1991]. The binding of the blocking antibody may change the conformation of syndecan-4 in such a way that its HS side-chain domains are no longer able to engage with the HS-binding site in the FGF receptor dimer [McKeehan et al., 1998].

To further understand FGF2-induced cell mineralization, the expression of the osteoblast-defining transcription factor Runx2 and the osteoblast marker ALP were investigated under osteogenic culture conditions. The results were consistent with the idea that FGF2 acts to stimulate osteoblast proliferation, thus ensuring that more osteoblasts can be calcified at later stages. At same time, FGF2 can also induce cell differentiation through an activation of Runx2 [Lian et al., 2004]. Except for a minor inhibitory effect at low concentrations, syndecan-4 antibody blockade seems to have no significant effect on either proliferation or Runx2 expression in osteogenic conditions. It is possible that some other mechanism mediated by syndecan-4, perhaps via focal adhesions [Woods and Couchman, 2001], is involved in the mineralization process.

A wide variety of functions have been ascribed to syndecan-4 in a wide variety of tissues. In lower vertebrates it has been reported to have a role in noncanonical Wnt signaling [Munoz et al., 2006]. Increased expression of syndecan-4 core protein in cardiac myocytes results in amplified production of nitric oxide by these myocytes in response to FGF2 [Li et al., 2002]. Coronary microvessels overexpressing syndecan-4 demonstrate increased relaxation responses to FGF2 compared to control microvessels in vitro [Li et al., 2002]. For most HSPGs it has been thought that the HS side-chains link growth factor to high-affinity receptor, thus forming a ternary complex [Rapraeger, 1995]. However, syndecan core proteins themselves also play an important role in signal transduction. Syndecan-4 possesses a unique sequence for phosphatidylinositol 4,5-bisphosphate-binding within its cytoplasmic tail [Lee et al., 1998; Horowitz et al., 1999] that is capable of efficiently activating PKC$\alpha$ [Oh et al., 1997; Simons and Horowitz, 2001]; such activity has been linked to the promotion of cell growth [Oh et al., 1997; Lallena et al., 1999; Besson and Yong, 2000]. That this activity is potent was shown in a human endothelial cell line engineered to overexpress syndecan-4; these cells could be driven to accelerated proliferation by FGF2, but not cells overexpressing syndecan-1 or glypican-1 [Volk et al., 1999]. Complementing this, inhibition of PKC activity prevented FGF2-dependent activation of endothelial cell growth [Kent et al., 1995]. This FGF2-triggered, syndecan-4–PKC pathway is thought to selectively regulate endothelial cell migration and proliferation [Horowitz et al., 2002], a mechanism that may also occur during FGF2-induced rat osteoblast proliferation and mineralization.

In conclusion, the results presented here demonstrate that FGF2 promotes syndecan-4 expression in rat primary osteoblasts, a secondary response requiring protein synthesis. Thus HSPGs may regulate osteoblast responses through multiple pathways.

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