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Fibroblast growth factor receptor 4 (FGFR4) expression in newborn murine calvaria and primary osteoblast cultures

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ABSTRACT Fibroblast growth factor receptor (FGFR) signalling is important in the initiation and regulation of osteogenesis. Although mutations in *FGFR1, 2,* and *3* genes are known to cause skeletal deformities, the expression of FGFR4 in bony tissue remains unclear. We have investigated the expression pattern of FGFR4 in the neonatal mouse calvaria and compared it to the expression pattern in cultures of primary osteoblasts. Immunohistochemistry demonstrated that FGFR4 was highly expressed in rudimentary membranous bone and strictly localised to the cellular components (osteoblasts) between the periosteal and endosteal layers. Cells in close proximity to the newly formed osteoid (preosteoblasts) also expressed FGFR4 on both the endosteal and periosteal surfaces. Immunocytochemical analysis of primary osteoblast cultures taken from the same cranial region also revealed high levels of FGFR4 expression, suggesting a similar pattern of cellular expression *in vivo* and *in vitro*. RT-PCR and Western blotting for FGFR4 confirmed its presence in primary osteoblast cultures. These results suggest that FGFR4 may be an important regulator of osteogenesis with involvement in preosteoblast proliferation and differentiation as well as osteoblast functioning during intramembranous ossification. The consistent expression of FGFR4 *in vivo* and *in vitro* supports the use of primary osteoblast cultures for elucidating the role of FGFR4 during osteogenesis.

KEY WORDS: *bone, osteoblast, osteogenesis, FGFR4, expression*

Introduction

Fibroblast growth factors (FGFs) are involved in controlling a variety of biological functions including mitogenesis, differentiation, chemotaxis, angiogenesis, and wound healing (Burgess *et al.*, 1989). Biological responses to FGF are mediated through four highly related receptor tyrosine kinases (FGFR1-4) coded by four distinct genes that have distinct but overlapping patterns of expression during development (Naski *et al.*, 1998). Alternative mRNA splicing of FGFR1, 2, and 3 leads to isoforms of these receptors that have unique ligand binding properties. Only recently has such splicing been identified in FGFR4 mRNA (van Heumen *et al.*, 1999). The FGFRs display varying affinities for each of the FGFs (a family of at least 23 polypeptides) and are expressed in a wide variety of tissues. Receptor-growth factor interactions are further modified by heparan sulfate proteoglycans (HSPG) (Lin *et al.*, 1999; Loo *et al.*, 2001; Nurcombe *et al.*, 2000). These molecules are required for FGF binding and appear to be necessary for formation of an active receptor-growth factor complex (Kan *et al.*, 1999; Spivak-Kroizman *et al.*, 1994).

The biological effects of FGF are mediated by intracellular signal transduction initiated by the growth factor-bound, activated FGFR. Mutations in the *FGFR 1, 2* and *3* genes have been linked to disorders in skeletal and cranial development (Webster *et al.*, 1997). However, no *FGFR4* related gene mutations have been linked to osteogenic disorders, although over expression of *FGFR4* has been associated with connective tissue fibrosis, presumably via excess fibroblast proliferation (Saito *et al.*, 2000).

Despite the high level of structural homology between the FGFRs, the expression pattern of FGFR4 differs significantly to that of FGFR1, 2, and 3. Initially the expression of FGFR4 was thought to be restricted to endodermal tissues and skeletal muscle (Korhonen *et al.*, 1992; Partanen *et al.*, 1991; Stark *et al.*, 1991; van Heumen *et al.*, 1999), whereas FGFR1-3 is expressed in high levels in foetal brain, calvarial bone, skin and growth plates of developing bones (Iseki *et al.*, 1999; Molteni *et al.*, 1999; Partanen *et al.*, 1991; Peters *et al.*, 1993; Peters *et al.*, 1992). More recently, FGFR4 expression has been identified in bone-marrow mesenchymal stem cell cultures, suggesting a role in the development of mesenchymal tissue (Walsh *et al.*, 2000). However, the precise

Abbreviations used in this paper: FGFR, fibroblast growth factor receptor; HSPG, heparan sulfate proteoglycan.

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role FGFR4 plays in the development of mesenchymal derived tissues is unknown.

In this investigation we examine the expression and localisation of FGFR4 in neonatal mice calvaria bone. Furthermore, we show that primary osteoblast cultures developed from neonatal mice show the same expression profile as the *in vivo* assay. This suggests that primary osteoblast cultures represent a viable model for examining FGFR4 function that is highly correlative with *in vivo* expression.

Results

Frozen Tissue Immunohistochemistry

To determine whether FGFR4 and osteopontin was present in the bone of 1-day-old murine calvaria, we performed immunohistochemistry using anti-FGFR4 and anti-osteopontin antibodies on frozen sections taken through the skullcap (Fig. 1 A,B,D). For consistency, only the parietal bone was assessed, although immunoreactivity was observed in the frontal and occipital bones. FGFR4 was highly expressed on the periosteal and endosteal surfaces of the skullcap, and throughout the rudimentary membranous bone sandwiched between these two layers. This reactivity was strictly localised to the cellular components (osteoblasts) between the periosteal and endosteal surfaces rather than the extracellular matrix. FGFR4 was also expressed in the developing bone regions in cells that appear to be close to (preosteoblasts) or within (osteoblasts) the newly formed osteoid. In addition, increased FGFR4 expression was observed on the endosteal (brain side) surface as compared with the periosteal (skin side) surface. FGFR4 was also highly expressed in the skeletal muscle covering the periosteal surface (data not shown). Unlike FGFR4, osteopontin was not expressed in the outermost cells of the periosteal or endosteal surfaces populated by replicating preosteoblast cells (Fig. 1D). Instead, osteopontin expression was found in the cells internal to these layers where the more mature osteogenic cells are found. This result confirms the presence of FGFR4 in rudimentary membranous bone, and further suggests this receptor has a role to play in preosteoblast biology (proliferation) and the deposition of osteoid on both the periosteal and endosteal surfaces.

Immunocytochemistry

Primary mouse osteoblasts were extracted from 1-day-old mice calvaria and cultured before being probed for the presence of osteopontin and FGFR4 (Fig. 1 E,F). FGFR4 immunoreactivity was present throughout the cell but particularly in the membrane and nuclear regions (as determined by a loss of reactivity when focusing down through the cell monolayer). No background staining of either osteopontin or FGFR4 substrate was observed (Fig. 1 E,F), nor was there reactivity in cells incubated with a FGFR4 blocking control peptide (Fig. 1G) or a non-immune IgG (data not shown). This staining is consistent with that observed for the immunohistochemical analysis and confirms the presence of osteopontin and FGFR4 in osteoblasts *in vivo* and *in vitro*. Furthermore, this reactivity was not changed by cellular extraction and purification or by cell culture and passaging (Fig. 1 B,D,E, and F).

Western Blot

To verify that cultured murine osteoblast cells express the FGFR4 and osteopontin proteins, cell lysates were electrophoresed in SDS-PAGE, followed by immunoblotting using FGFR4 and osteopontin antisera (Fig. 2 A,B). In this analysis, a FGFR4 protein of 120 kDa was

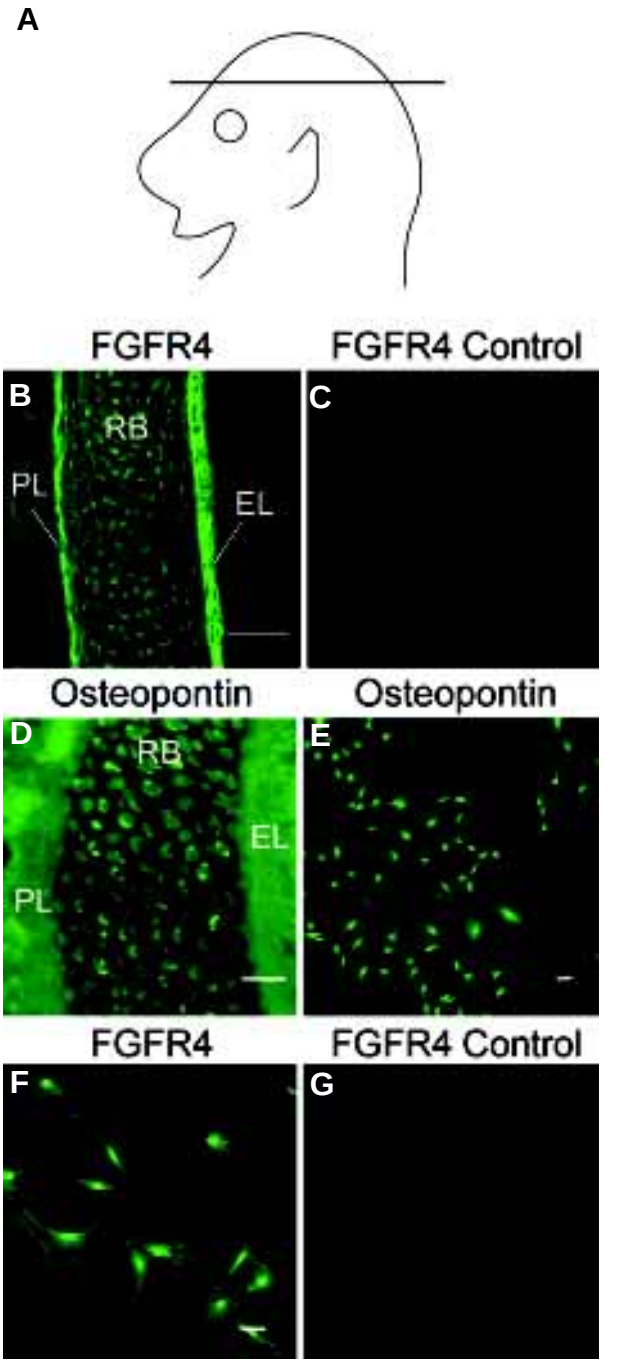

Fig. 1. FGFR4 and osteopontin is expressed in murine bone and bone cell cultures. (A) *Schematic representation of a 1-day-old murine calvaria. Expression and localisation of FGFR4* **(B)** *and osteopontin* **(D)** *in the murine scullcap and primary osteoblast cell culture* **(F,E)**. *Frozen sections were taken through the parietal bone (solid line) (A), and used for immunohistochemistry (B,D). Frozen sections (B,D) showed rudimentary bone (RB) sandwiched between an outer periosteal layer (PL) and an inner endosteal layer (EL). Primary osteoblast cell cultures (F,E) stained positively for FGFR4 and osteopontin, both within the nucleus and the cellular cytoplasm. Both tissue sections and osteoblast cultures were probed with streptavidin-FITC and examined using confocal microscopy. Incubation with appropriate FGFR4 blocking peptide (C,G) showed no immunoreactivity. Bar represents 100 μm in B,D and 20 μm in E,F.*

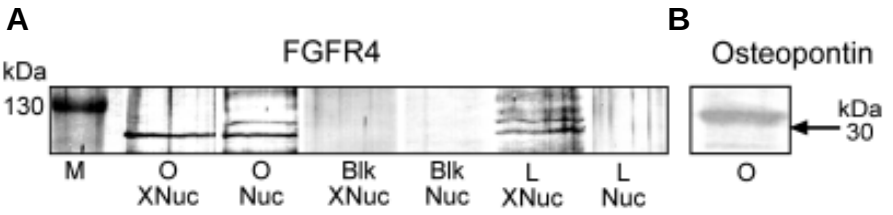

Fig. 2. Western blot analysis confirming *in vitro* expression of FGFR4 and osteopontin. *Osteoblast cells (O) extracted from 1-day-old murine calvaria were cultured in standard medium until confluent. Liver samples (L) from the same animals were used as controls.* **(A)** *Protein extracted from both the nuclear (Nuc) and extra nuclear (XNuc) compartments was run on a 8% polyacrylamide gel and transferred to PVDF. The blots were probed with antisera against FGFR4 or an excess of blocking peptide (Blk) pre-incubated with FGFR4 antisera.* **(B)** *Protein extracts were also blotted with antisera against osteopontin to confirm an osteoblast phenotype. Lane (M) is molecular weight marker (5 μl).*

detected in lysates from both the nuclear and extra nuclear osteoblast fractions, whilst in the liver control samples, only the extra nuclear fraction showed immunoreactivity for FGFR4. In both samples the immunoreactivity was blocked by incubation with a control peptide (Fig. 2A) and non-immune IgG (data not shown). This result confirms the presence of the FGFR4 protein in 1-day-old murine primary osteoblasts as shown by immunocytochemistry and immunohistochemistry. Furthermore, the FGFR4 nuclear staining observed by immunocytochemistry was confirmed by Western blots of the nuclear extracts.

RT-PCR

To further examine the expression of FGFR4, transcripts were studied by PCR amplification of cDNA derived from primary osteoblast cultures and liver control samples. RT-PCR analysis revealed that osteoblast cultures and liver samples express abundant mRNA encoding FGFR4, with an intense band of the expected size (243 bp) observed (Fig. 3). GAPDH was expressed in both osteoblasts and liver samples (267 bp) and no bands were observed in either sample for the reverse transcriptase and RNA controls (data not shown). This result confirms that FGFR4 mRNA signals parallel FGFR4 protein expression in both primary osteoblasts and liver samples.

Discussion

In this study we examined the *in vivo* and *in vitro* expression and localisation of FGFR4 and osteopontin in 1-day-old murine calvaria and primary osteoblast cultures. Unlike previous studies that failed to show FGFR4 expression in bone (Iseki *et al.*, 1999; Partanen *et al.*, 1991; Peters *et al.*, 1992), we found high levels of expression within regions of newly formed bone (osteoid) and in osteoblasts sandwiched between the inner endosteum and outer periosteum (Fig. 1B). We suggest the intense expression of FGFR4 in the endosteal and periosteal regions is due to its active role in controlling the osteoprogenitor cell population, consistent with recent findings by Walsh *et al.* (2000). Although the four FGFRs are structurally similar, the spatiotemporal expression pattern of FGFR4 has been shown to differ significantly to that of FGFR1, 2, and 3. High levels of FGFR4 have been found in liver, lung, kidney, and skeletal muscle (Korhonen *et al.*, 1992; van Heumen *et al.*, 1999), whereas no FGFR4 has been detected in calvarial bone, skin or growth plates of developing bones, all of which highly express FGFR1, 2, and 3 (Iseki *et al.*, 1999; Molteni *et al.*, 1999; Partanen *et al.*, 1991; Peters *et al.*, 1993; Peters *et al.*, 1992). Our finding that FGFR4 is expressed in the osteoblasts of rudimentary calvarial bone and a lack of expression in the extracellular matrix therefore represents a novel finding. In addition, we confirm that osteopontin expression is restricted to the more differentiated osteoblasts sandwiched between the inner and outer osteogenic layers, as previously reported by Iseki *et al.* (1997, 1999).

FGFR1 and 2 have been implicated in the functional control of osteoblast cells by FGFs, and appear at the onset of osteogenesis in progenitor cells (Molteni *et al.*, 1999). These receptors are thought to be involved in maintaining the proliferation-differentiation balance principally through FGFR2 regulation of osteoblastic proliferation and FGFR1 regulation of osteogenic differentiation (Iseki *et al.*, 1997; Iseki *et al.*, 1999).

In contrast, FGFR3 is a negative regulator of bone growth controlling the proliferation of chondrocytes involved in endochondral ossification (Colvin *et al.*, 1996; Deng *et al.*, 1996; Molteni *et al.*, 1999). Consistent with this functional role, activating mutation in the genes encoding these receptors have been linked to skeletal abnormalities in humans (Muenke *et al.*, 1995). However no such abnormalities have been linked to mutation in the FGFR4 gene despite FGFR4 sharing 56-60% homology to the other FGFRs at the protein level (Gaudenz *et al.*, 1998). Gaudenz *et al.* (1998) suggested that FGFR4-link mutations did not result in skeletal abnormalities because this receptor was involved in the development of endoderm derivatives along with myogenic genes and not associated with bone tissues. Here we have shown that not only do osteoblasts in the rudimentary bone express high levels of FGFR4, but that FGFR4 is also highly expressed in the osteogenic membranes where osteogenic stem cells are undergoing proliferation and differentiation during intramembranous ossification. Taken together, these observations suggest that FGFR4 may regulate the rate at which osteogenic cells progress through the phenotypic stages of recruitment, proliferation and differentiation.

Cultures of primary mouse calvarial osteoblasts also express high levels of FGFR4 and osteopontin as shown by immunocytochemistry, immunoblotting and RT-PCR. This suggests that primary osteoblasts do not alter their phenotype nor lose their FGFR4 expression following extraction and expansion by tissue culture. As both FGFR4 mRNA transcripts and transmembrane proteins were identified, this receptor may be involved in osteoblastic cell function in a manner not yet identified. Therefore, the contention that FGFR4 is not present in bony tissue and that mutations do not produce skeletal abnormalities seems highly unlikely. Rather, it is possible that unidentified mutations in FGFR4 might account for such abnormalities.

More recently FGFR4 expression has been identified in osteo-

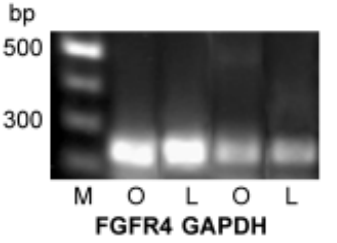

Fig. 3. mRNA transcripts of FGFR4 are present in cultured bone cells. *Total RNA was extracted from primary mouse osteoblast cultures and liver samples, reverse transcribed and specific PCR was carried out as described. The individual products were electrophoresed in 3% agarose gels. Lane (M) base pair ladder marker, (O) primary osteoblast, (L) liver sample for both FGFR4 and GAPDH specific primers.*

genic cells (Chikazu *et al*., 2000; Walsh *et al*., 2000), although this was limited to an *in vitro* assay. The expression pattern of this receptor during bone development clearly needs to be fully examined and its precise role in osteoblast biology clarified.

Materials and Methods

Antibodies

A rabbit polyclonal antibody with its epitope mapping to the carboxy terminus of FGFR4 from human origin (identical to the corresponding mouse sequence) was obtained from Santa Cruz (California, USA). The peptide used to raise the FGFR4 antibody (blocking peptide) and a non-immune rabbit IgG was also obtained from Santa Cruz (California, USA) and used as negative controls. Antibody neutralisation was performed by pre-incubating the primary antibody in an excess of blocking peptide for 2 h at 22°C. Biotinylated anti-rabbit and anti-rat IgM, and ABC Elite were purchased from Vector Labs (Peterborough, UK). A rat osteopontin polyclonal antibody and streptavidin–conjugated fluorescein isothiocynate (FITC) were obtained from Chemicon (California, USA). FITC staining was observed using confocal microscopy (Bio-Rad MRC 1024, CA, USA). All images were taken with a x10 objective and captured at 1024 x 1024 pixels. Montages were prepared with the use of Photoshop 6.0 (Adobe, Mountain View, CA).

Animals

One-day-old Quackenbush mice were obtained from the University of Queensland Animal Production Department. Animals were euthanased, the calvaria removed and the skin excised to release the skullcap. Brain tissue was removed and the scullcap washed several times in sterile phosphate-buffered saline (PBS) pH 7.4 (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl). The cleared scullcaps were either frozen immediately in tissue-tek O.C.T compound (Miles, USA) in liquid N_2 or further processed for cell culture. Liver samples free of surrounding connective tissue were also removed and immediately frozen in liquid N_2 and stored at –70°C until further processing.

Frozen Tissue Immunohistochemistry

Glass slides were cleaned with 95% EtOH, treated with subbing solution and allowed to air-dry. Cryostat sections 10 μm thick were adhered to slides and allowed to air-dry. The sections were rinsed with TBST (100mM Tris-HCl (pH 7.5), 0.9% NaCl, 0.1% Tween-20) containing 0.5% Triton X-100 for 5 min and non-specific binding sites blocked with normal rabbit serum diluted 1:5 in TBST for 20 min. Sections were then incubated in FGFR4 or osteopontin primary antibody diluted 1:40 in TBST with 1% normal rabbit serum overnight at 4°C, washed, and incubated with secondary antibody diluted 1:100 in TBST for 1 h at 22°C. Sections were then incubated with streptavidin–FITC diluted 1:100 in TBST, for 1 h at 22°C, washed and mounted in PBS:glycine (1:1). All steps were carried out in a humid, light-proof chamber at 22°C. Slides were then examined by confocal microscopy.

Cell Culture

Skullcaps were collected and the parietal bones harvested, well clear of the developing sagittal suture, and digested in PBS containing 0.1% collagenase and 0.2% dispase (0.2 μm filter sterilised) at 37°C for 10 min. The solution was removed to a fresh sterile tube (fraction 1). This procedure was repeated with fresh solution five more times (fractions 2-6). Fractions 2-6 were combined and the cells pelleted by centrifugation at 5000 x g for 5 min. Cells were seeded at $3x10^5$/25 cm^2 in Nunclon T25 cell culture flasks (Nunc, Denmark) in 5% CO_2 at 37°C in 10 mM/L Hepes-buffered modified Eagles medium with Earl's salts (EMEM) (Life Technologies, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Sigma, St. Louis, MO), 2 mM/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Paisley, Scotland) (herein after referred to as standard medium). After 3-4 days, the cells were detached with 0.25% trypsin 0.5mM EDTA (Life Technologies, Paisley, Scotland) and passaged in standard medium or aliquotted to freezing medium at $1x10^6$ cells/mL and stored in liquid N_2.

Immunocytochemistry

Cells were seeded at $2x10^4$ in 500 μl on to poly-L-lysine (Sigma, St. Louis, MO) coated 12 mm grade 1 sterile glass coverslips in standard medium at 5% CO_2 and 37°C and allowed to adhere (2 h). Cells were rinsed in PBS, fixed with fresh 4% paraformaldehyde in PBS for 0.5 h at 22°C, washed, and non-specific binding sites blocked for 1 h in blocking buffer (PBS containing 0.1% Tween-20, 0.5% Triton X-100). Cells were then incubated in FGFR4 or osteopontin primary antibody diluted 1:40 in blocking buffer for 1 h at 22°C, washed, incubated with the appropriate secondary antibody diluted 1:100 in blocking buffer, washed, and incubated in streptavidin-FITC diluted 1:100 in blocking buffer for 1 h. After staining, the coverslips were mounted in Mowiol (Calbiochem, San Diego, CA) and viewed by confocal microscopy.

Western Blot Analysis

After the cultures reached confluence the cells were passaged once, and upon reaching confluence again, washed in PBS and lysed with ice-cold lysis buffer (1% Triton X100, 150 mM NaCl, 10 mM Tris pH 7.4, 2 mM EDTA, 0.5% NP 40, 0.1% SDS) containing protease inhibitors (1 mM sodium orthovanadate, 10 ug/mL leupeptin, 1 ug/mL aprotinin and 1 mM PMSF). Positive control samples of liver were also lysed as described above using a mortar and pestle. The protein concentration in the osteoblast and liver supernatants was determined using a Protein Assay Kit (Bio-Rad, CA, USA), based on the method of Bradford (Bradford, 1976). Aliquots of the supernatants (30 μg) were boiled for 5 min in an equal volume of 2 x Laemmli sample buffer (Laemmli, 1970) and cellular proteins separated on 8% SDS-PAGE (polyacrylamide gel electrophoresis) mini-gels and transblotted onto Hybond-P membranes (Amersham, Buckinhamshire, UK). Nonspecific binding sites were blocked (1% BSA in TBST) for 1 h at 22°C. Membranes were then incubated with either FGFR4 or osteopontin primary antibody diluted 1:1000 in TBST overnight at 4°C, washed, then incubated for 1 h with secondary antibody diluted 1:2000 in TBST, washed, and incubated in ABC Elite for 0.5 h. Following washes in TBST then TBS the reactivity was determined by the ECLplus chemiluminescence reaction (Amersham, Buckinhamshire, UK) and visualised at 700 nm using a dual-line laser system (Fluorimager 595, Molecular Dynamics).

Nuclear Fractionation

FGFRs have a membrane-bound localisation; however to examine the presence of FGFR4 in the nucleus, cell nuclear extracts from both osteoblasts and liver samples were prepared as described by Herrmann *et al.* (1997). Osteoblast cells and liver samples were trypsinized as above and pelleted in EMEM containing 10% FCS. After washing with ice-cold PBS, lysates were repelleted and resuspended in 400 μl of ice-cold hypotonic buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 3 mM PMSF, and 3 mM DTT). After 10 min on ice, 25 μl of 10% Nonidet P-40 was added and crude nuclei were collected by centrifugation for 5 min. The nuclear pellet was resuspended in high salt buffer (50 mM Hepes, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 3 mM DTT, and 3 mM PMSF). After 30 min on ice with frequent agitation, the insoluble nuclear material was pelleted in a microcentrifuge for 10 min at 4°C. Crude nuclear protein was collected from the supernatant and snap-frozen in a dry ice/ethanol bath. After thawing and boiling for 5 min in Laemmli buffer, the nuclear extracts were subjected to SDS-PAGE and probed with FGFR4 antisera as above.

RNA Preparation

Total RNA was isolated from primary osteoblast cultures and liver samples using an RNeasy mini kit (Qiagen, Hilden, GR) according to manufacturer's instructions. The concentration and purity of RNA was determined by measuring the A_{260} and A_{280} absorbency in a spectrophotometer ($GeneQuant_{pro}$, Amersham, Buckinhamshire, UK).

Oligonucleotide Primers

Oligonucleotide primers used to amplify FGFR4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed against the mouse cDNA sequences as follows: FGFR4 (X59927)

sense, 5'-GTA CCC TCG GAC CGC GGC ACA TAC-3'; antisense, 5'-GCC GAA GCT GCT GCC GTT GAT G-3' (Hsu *et al.*, 2001), GAPDH (M32599) sense 5'-ACT TTG TCA AGC TCA TTT CC-3'; antisense 5'-TGC AGC GAA CTT TAT TGA TG-3' (Boudreau *et al.*, 1999) and purchased from Genset Pacific (Lismore, NSW, Australia). PCR fragments are 243 and 267 base pairs for FGFR4 and GAPDH respectively. The FGFR4 oligonucleotides were a kind gift by Dr. James Pickles, School of Biomedical Sciences, Vision, Touch and Hearing Research Centre, University of Queensland, Brisbane, Australia and are based on the oligonucleotides described in Brickman *et al.* (1995), the product of which was subcloned into pGem3zf (Promega, Madison WI) to verify the identity.

RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) were performed sequentially in the same reaction tube using a one-step RT-PCR kit (Qiagen, Hilden, GR) according to manufacturer's instructions. RT-PCR was performed in a thermal cycler (PCR Express, Hybaid, Middlesex, UK) and approximately 2 μg of primary osteoblast and liver mRNA reverse-transcribed at 50°C for 30 min to generate cDNA. PCR was performed for 35 cycles, with 50 s at 94°C for denaturation, 50 s at 62°C for annealing and 60 s at 72°C for extension. Possible contamination by genomic DNA was determined by two means - amplimer pairs anneal in regions encoded by separate exons (no band corresponding to genomic DNA being seen in any case), and secondly, all reactions were also run with samples omitting the reverse transcriptase (-ve RT), or RNA (-ve RNA), which were replaced with water, none of which produced detectable reaction products.

Acknowledgements

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