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Abnormal Bone Growth and Selective Translational Regulation in Basic Fibroblast Growth Factor (FGF-2) Transgenic Mice

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Basic fibroblast growth factor (FGF-2) is a pleiotropic growth factor detected in many different cells and tissues. Normally synthesized at low levels, FGF-2 is elevated in various pathologies, most notably in cancer and injury repair. To investigate the effects of elevated FGF-2, the human full-length cDNA was expressed in transgenic mice under control of a phosphoglycerate kinase promoter. Overexpression of FGF-2 caused a variety of skeletal malformations including shortening and flattening of long bones and moderate macrocephaly. Comparison by Western blot of FGF-2 transgenic mice to nontransgenic littermates showed expression of human FGF-2 protein in all major organs and tissues examined including brain, heart, lung, liver, kidney, spleen, and skeletal muscle; however, different molar ratios of FGF-2 protein isoforms were observed between different organs and tissues. Some tissues preferentially synthesize larger isoforms of FGF-2 while other tissues produce predominantly smaller 18-kDa FGF-2. Translation of the high molecular weight isoforms initiates from unconventional CUG codons and translation of the 18-kDa isoform initiates from an AUG codon in the FGF-2 mRNA. Thus the Western blot data from the FGF-2 transgenic mice suggest that tissue-specific expression of FGF-2 isoforms is regulated translationally.

INTRODUCTION

Our main goals in producing FGF-2 transgenic mice were to identify the target cells and tissues for the effects of FGF-2 overexpression and to produce an experimental mouse to study translational regulation of FGF-2 expression. Prediction of phenotypes for FGF-2 transgenic mice is difficult because of the wide variety of reported functions for FGF-2 (Basilico and Moscatelli, 1992). Target tissues for constitutive overexpression of FGF-2 may include mesodermal derivatives and neuronal tissues in development because they normally express endogenous FGF-2 (Gonzalez *et al.*, 1990) and FGF-2 is reported to be a potent mesodermal inducer (Kimelman and Kirschner, 1987; Krah *et al.*, 1994; Slack and Isaacs, 1994). FGF-2 has been reported to affect growth and development of nerve (Eckenstein, 1994), endothelium (Montesano *et al.*, 1986), skeletal muscle (Olwin *et al.*, 1994), cardiac muscle (Schneider and Parker, 1991), smooth muscle

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(Lindner and Reidy, 1993), and cartilage (Trippel *et al.*, 1993), any of which may be affected by transgenic FGF-2. Among pathologies, FGF-2 is reported to be neurotrophic (Gomez-Pinilla *et al.*, 1992; Macmillan *et al.*, 1993), and many tumors produce FGF-2 (Nguyen *et al.*, 1994) that may be neoplastic (Rifkin and Moscatelli, 1989) and function as an angiogenic factor (Folkman and Klagsburn, 1987). FGF-2 has also been found in the heart where it may affect growth and survival of cardiomyocytes and growth of the coronary blood vessels during hypertrophy and ischemia (Kardami and Fandrich, 1989; Yanagisawamiwa *et al.*, 1992). Accelerated regrowth of endothelium in balloon-catheterized arteries (Lindner *et al.*, 1990) and accelerated wound healing are also attributed to FGF-2 (Kurita *et al.*, 1992). Based on these reports, we suspected that FGF-2 overexpression would have dramatic effects on the nervous and vascular systems, possibly causing a neuropathy, aberrant neovascularization, or possibly predisposing FGF-2 transgenic mice to malignant neoplasms.

Expression and function of FGF-2 receptors (FGFR) provide other clues to the target organs for FGF-2 in development and disease. FGF proteins bind with high affinity to cell surface proteins or low affinity to extracellular matrix proteins. The genes for five high affinity FGF binding proteins have been cloned and characterized. FGFR1–4 contain cytoplasmic tyrosine kinase domains that activate signal transduction (Jaye *et al.*, 1992). Several splice variant FGFR are generated from these genes with differential affinities for the FGF ligands and they are differentially expressed by nerve and mesodermal derivatives (Patstone *et al.*, 1993; McKeehan and Kan, 1994). The other cell surface, high affinity FGF binding molecule known as the "cysteine-rich FGFR" does not contain a tyrosine kinase domain and its mechanism of action is unknown (Olwin *et al.*, 1994). Currently, the proposed mechanism for function of the FGFs is binding of the ligand and a heparin sulfate co-factor to the target cell surface tyrosine kinase receptor (Yayon *et al.*, 1991) that stimulates signal transduction, which activates nuclear transcription factors resulting in cell growth and/or differentiation (Spivak-Kroizman *et al.*, 1994). Mutations in FGFR1–3 have recently been associated with skeletal abnormalities in humans. Mutation of FGFR-1 causes Pfeiffer syndrome (Muenke *et al.*, 1994), mutations in FGFR-2 cause Jackson-Weiss and Crouzon syndrome (Jabs *et al.*, 1994; Reardon *et al.*, 1994), and mutations in FGFR-3 cause achondroplasia or thanatophoric dysplasia (Rousseau *et al.*, 1994; Shiang *et al.*, 1994; Tavormina *et al.*, 1995). One FGFR has been deleted by homologous recombination using ES cells in "gene targeting" experiments. Loss of the FGFR-1 gene results in embryonic lethality in null FGFR-1 mice (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). The FGFR data suggested that overexpression of FGF-2 would most dramatically affect the skeletal system. This proved to be the most accurate indicator of the actual phenotype in FGF-2 transgenic (TgFGF214) mice.

FGF-2 may, however, have other activities beyond its cell surface, receptor-mediated functions. Translational regulation from alternative CUG translation start sites upstream from the AUG is reported as a means to control expression of FGF-2 isoforms in humans and rodents (Florkiewicz and Sommer, 1989). Translation from the human CUG sites produces 24-, 23-, and 22-kDa isoforms while translation from the AUG produces 18-kDa FGF-2 in all species (Florkiewicz *et al.*, 1991b). All of the FGF-2 isoforms lack a classical signal peptide for trafficking through the ER/golgi secretory pathway (Abraham *et al.*, 1986). The high molecular weight FGF-2 isoforms localize to the nucleus while the 18-kDa isoform is predominantly cytoplasmic or extracellular (Brigstock *et al.*, 1990; Florkiewicz *et al.*, 1991a). The 18-kDa isoform is believed to be exported independently of the ER golgi pathway (Florkiewicz *et al.*, 1995) and predominates in FGF-2 bound to the extracellular matrix (Brigstock *et al.*, 1991). The nuclear forms of FGF-2 can also be phosphorylated, leading to consideration of FGF-2 as a transacting nuclear factor, regulated by phosphorylation/dephosphorylation (Vilgrain and Baird, 1991). It is unclear whether different biological functions exist for the nuclear and cytoplasmic forms of FGF-2 and the effect of FGF-2 on a cell or in a tissue may result from either nuclear and cell surface (FGF ligand/receptor) activities or a pleiotropic balance between the two. This suggests that different tissues or cells may be utilizing the nuclear or cytoplasmic FGF-2 in different ways during morphogenesis and in disease. Our goal was to produce transgenic mice for experiments to study translational regulation of FGF-2 proteins.

We made three stable lines of FGF-2 transgenic mice designated TgFGF214–15, TgFGF214–16, and TgFGF214–48. The primary effects of FGF-2 overexpression in all three lines were on bone morphogenesis where macrocephaly and chondrodysplasia were evident. Western blots for FGF-2 on brain, heart, lung, liver, kidney, slpeen, and skeletal muscle all show increased expression of FGF-2, but the isoform profiles differed between tissues, suggesting tissue-dependent isoform-specific translation regulation of FGF-2 expression.

MATERIALS AND METHODS

Production of Transgenic Mice

A constitutive phosphoglycerate kinase promoter and polyadenylation sequence were used for general overexpression of FGF-2 (a gift from Dr. Mike McBurney). A human FGF-2 cDNA, that included coding sequences for all three CUG and the AUG translation start sites (Shibata *et al.*, 1991), was ligated to the promoter with a short

intronic sequence (Choi *et al.*, 1991) inserted between the promoter and the FGF-2 cDNA in plasmid Bluescript II SK (Stratagene, La Jolla, CA). This construct, TgFGF214, was removed from the plasmid by digestion with *Xba*I and *Cla*I, separated from impurities in 1% low melting point agarose by electrophoresis, and purified with Gelase (Epicenter Technologies, Madison, WI) for microinjection.

FVB/N mice (Taconic Farms, Germantown, NY) were used for pronuclear injections and resulting TgFGF214 transgenic mice were maintained in a pathogen-free environment according to Public Health Service and Institutional standards. Pronuclear injection of TgFGF214 was conducted using methods similar to those of Hogan *et al.*, 1986. Founder mice containing the transgene were bred to nontransgenic littermates. Thereafter, all transgenic mice were continued as inbred lines.

Pathology

Histologic analyses of transgenic and nontransgenic littermates were conducted using standard methods. Transgenic and nontransgenic littermates were weighed and examined externally for lesions or gross anatomical abnormalities, then euthanized and dissected for gross examination of all internal organs and tissues. After complete gross dissection, carcasses were skinned and fixed in 70% ethanol. Viscera were fixed in 10% neutral buffered formalin in preparation for paraffin sectioning at 5 microns. Samples of the following organs were sectioned from selected transgenic and nontransgenic animals: brain, eye, conjunctiva, Harderian gland, heart, lung, trachea, thymus, liver, gall bladder, pancreas, spleen, gastrointestinal tract, kidney, adrenal, urinary bladder, mediastinal and submandibular lymph nodes, salivary gland, diaphragm, masseter and longissimus dorsi muscles, gonads, and the long bones of one extremity. Hematoxylin and eosin–stained slides were evaluated by light microscopy. For FGF-2 immunocytochemistry, TgFGF214 mice and nontransgenic littermate controls were anesthetized with metophane, perfused transcardially with 10% formalin/phosphate-buffered saline (PBS), and post-fixed for 2 h at 4°C. Immunostaining for FGF-2 was conducted on paraffin sections according to the method of Gonzalez *et al.*, (1990). Briefly, the sections (8 um thick) were deparaffinated, hydrated through descending concentrations of ethanol, rinsed in 50 mM Tris, 0.9% NaCl (TBS) containing 0.3% Tween 20 (Sigma, St. Louis, MO). Nonspecific binding was blocked by incubating the tissue sections with 3.0% normal donkey serum (Jackson Immunoresearch Labs, West Grove, PA). Sections were then incubated overnight with anti-FGF-2 antibodies (RB773; Gonzalez *et al.*, 1990) diluted in TBS containing 0.3% Tween-20 and 3% bovine serum albumin. Slides were rinsed and the endogenous peroxidase was quenched by treating the sections in 0.5% hydrogen peroxide in TBS for 30 min. The tissue sections were incubated in donkey biotinylated anti-rabbit (Jackson Immunoresearch), followed by ABC complex (Vector, Burlingame, CA). Finally the sections were treated with 0.5% diaminobenzidine (DAB; Sigma) in TBS containing 0.01% hydrogen peroxide. The DAB-tinted sections were rinsed, counterstained with hematoxylin, dehydrated, and protected with coverslips.

Gross examination of the FGF-2 transgenic mice showed abnormalities in the skeletal system; as a result, a more detailed analysis was undertaken. Skeletons were evaluated for gross deformities after maceration in potassium hydroxide and staining with alizarin red-S. Limb growth in the transgenic mice and nontransgenic littermate controls was assessed by measurement of the long bones. Limbs were removed and autoclaved to facilitate removal of the soft tissue. The long bones of the remaining skeleton were dried at room temperature and measured with a ruler under a dissection microscope to the nearest 0.5 mm. Statistical analysis consisted of an analysis of variance between lengths of bones from the transgenic and nontransgenic groups.

The skeletal abnormalities suggested that the TgFGF214 mice may have a general growth problem leading to runting. Longitudinal growth curves to monitor the weight gain of the three lines of TgFGF214 mice were constructed by weighing pups for the first 60 days following weaning. Thereafter, the mice were weighed on a weekly basis up to 36 wk of age.

Evaluation of the cardiovascular system consisted of morphological evaluation of sectioned tissues for aberrant neovascularization and an evaluation of vascular smooth muscle cell (VSMC) hypertrophy. To test for hypertrophy, aortae from FGF-2 transgenic mice and their nontransgenic littermates (ages 8–12 wk) were explanted on collagen-coated cultured dishes for outgrowth of VSMCs as previously described (Dorn *et al.*, 1987). Cells were passaged by trypsinization and split at a 1:4 dilution. The current study used cells from passages 4 and 6. The smooth muscle phenotype of the cultured cells was verified by immunostaining of α smooth muscle actin.

For determination of VSMC DNA and protein content, cells were plated in 6-well culture dishes at a density of 105 cells per well and growth arrested by withdrawal of serum from the media for 48 h. Growth arrest was confirmed by assessing incorporation of [^{3}H]thymidine and [^{3}H]leucine (Dorn *et al.*, 1992). DNA and protein were simultaneously assayed using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Briefly, quiescent cells monolayers were lysed in situ with 1 ml Tri-Reagent and the lysate was phase separated with chloroform. DNA was precipitated from the organic and interphase with ethanol and quantified by UV absorbance at 260 nm. Protein was isolated from the phenol/ethanol supernatant by isopropanol precipitation, solubilized in 1% SDS, and quantified using a Bradford assay. Bovine serum albumin in 1% SDS was used as a protein standard.

Genetic Analysis

Southern blots of genomic DNA samples from founder mice and offspring were conducted to confirm the presence of the transgene and to assess relative doses of the transgene among founders. A short segment of the tail from each founder mouse was removed with scissors, digested, and processed to isolate genomic DNA (Laird *et al.*, 1991). The concentration of each genomic DNA sample was assessed by measuring absorbance at 260 nm. Ten-microgram quantities of genomic DNA from each founder mouse were digested overnight with *Eco*RI (United States Biochemical, Cleveland, OH) at 37°C and separated by horizontal gel electrophoresis with 0.8% agarose in Tris/acetate-EDTA buffer. Following electrophoresis, the gel was stained with ethidium bromide (10 μg/ml TAE) and photographed. The DNA was capillary transferred to a Nytran Plus membrane (Schleicher & Schuell, Keene, NH) that was baked for 2 h at 60°C, and prehybridized and hybridized according to standard methods (Church and Gilbert, 1984). A probe for hybridization to label the transgene was made from an *Eco*RI/*Not*I fragment of the original human FGF-2 clone by multi-prime labeling with the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA) in the presence of [α-^{32}P]dCTP according to the manufacturer. Autoradiographs were made by exposure of the blot to x-ray film.

After initial characterization of the founder mice and the first (F1) generation of mice by genomic Southern blot, all animals were genotyped using the polymerase chain reaction (PCR). Tissue samples from litters were digested in a buffer containing 10 mM Tris-Cl (pH 7.4), 0.01 mM EDTA, 0.5% NP-40, 0.5% Tween-20, 1 U/μl proteinase K (Sigma) overnight at 50°C. The samples were heated at 100°C for 10 min to inactivate the proteinase K. Two microliters of genomic DNA were added to a PCR reaction mix containing 10 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$, dNTPs (Pharmacia, Milwaukee, WI), and primers. Two separate sets of primers were used. The first primer set was specific to the transgene with the forward primer (5′)CTTCAAAAGCGCACGTCTGC(3′) in the phosphoglycerate kinase promoter and the reverse primer (5′)GCCTGCCACACCTCAAGCTT(3′) in the intron. The second primer set was derived from sequences in the mouse TGF-β gene (Shull *et al.*, 1992), and used as an in-lane positive control. The reagents, excluding *Taq* polymerase, were mixed, overlaid with mineral oil, then placed in a

thermal cycler (Ericomp, San Diego, CA) and heated to 95°C for 3 min. After cooling to 80°C, the 2U *Taq* polymerase (Life Technologies, Grand Island, NY) and an aliquot of water were added to bring the reaction mix to 20 μl total volume. The reaction was allowed to proceed for 30 cycles as follows: 95°C for 1 min, 65°C for 2 min, 72°C for 3 min, and a final cycle of 72°C for 15 min. The reactions were separated in 1.2% agarose by horizontal gel electrophoresis in TAE, stained with ethidium bromide, and photographed under UV light.

RNA Analysis

Total RNA was isolated from TgFGF214 mice and nontransgenic littermate controls using RNasol according to the manufacturer (Biotex, Houston, TX). RNase protection was designed to examine expression from the five-prime region of the human transgene. Probes for RNase protection were derived from either human FGF-2 (Florkiewicz and Sommer, 1989) or rodent cyclophilin (DePaolo *et al.*, 1992; a gift from Dr. S. Shimasaki) cDNA subclones in pBS (Stratagene). The human FGF-2–specific anti-sense riboprobe spans nucleotides (nts) +123 through +339 relative to the transcription start site (Shibata *et al.*, 1991), including sequences corresponding to the first eight amino acids of the 24-kDa isoform. All templates for anti-sense riboprobes were linearized with the appropriate restriction enzyme, and then gel purified using Geneclean (BIO 101, La Jolla, CA). Radiolabeled antisense transcripts were prepared in the presence of [^{32}P]uridine triphosphate using the appropriate RNA polymerase. The protected probe length for human FGF-2 is 140 nts and 128 nts for cylcophilin. The RNase protection assay has been previously described in detail (Melton, 1984; Singer *et al.*, 1990). Briefly, total RNA (10 μg) was hybridized for 18 h at 56°C with ^{32}P-labeled antisense RNA probe in 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer (pH 6.4) containing 400 mM NaCl 1 mM EDTA and 80% formamide. Yeast tRNA (10 μg) was added to each sample as a nonspecific negative control. Excess probe and nonhybridized RNA was removed by RNase digestion (50 μg/ml RNase A and 50 U/ml RNase T1) in 10 mM Tris (pH 7.5), 300 mM NaCl, 5 mM EDTA for 1 h at 30°C, followed by incubation with proteinase K (200 μg/ml) in 2% SDS for 30 min at 37°C. Samples were phenol chloroform extracted, ethanol precipitated, and dissolved in formamide sample buffer, then fractionated by denaturing SDS-PAGE followed by autoradiography.

Reverse-transcription (RT)-PCR was conducted on the same RNA samples that were used for RNase protection. Two micrograms of total RNA were reverse transcribed using Perkin-Elmer kit #808–0017 (Norwalk, CT) according to the manufacturer. Following reverse transcription, 5 μl was amplified using either FGF-2 primers [forward, (5′)AAGAGCGATCCGCACACTAA(3′); reverse, (5′)-GGATAGCTTTCTGTCCAGGT(3′)] or β-actin primers [forward, (5′)GTCGCCCTGGACTTCGAGC(3′); reverse, (5′)GGTACATGGT-GGTGCCGCCA(3′)] as controls. Amplification was carried out using Ampliwax beads according to the manufacturer (Perkin-Elmer) in a Perkin-Elmer thermal cycler with the following program: 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for 35 cycles. Equal aliquots of each sample were analyzed with a 1% agarose gel and stained with ethidium bromide for photography.

Western Blot Analysis

Tissue samples from transgenic and nontransgenic littermates of founders and their offspring were analyzed for human FGF-2 protein expression by Western blot. Standards for the multiple human and rodent FGF-2 isoforms were prepared from extracts of COS-1 cells transfected with expression vectors encoding human or rat FGF-2 as previously described (Florkiewicz and Sommer, 1989). To prepare extracts for Western blots, samples were dissected, rinsed once in PBS, frozen in liquid nitrogen, and stored at −70°C. Frozen samples were homogenized in cold extraction buffer (1% NP-40 [Calbiochem, La Jolla, CA], 0.5% deoxycholate [Sigma], 20 mM Tris [pH 7.5], 5 mM EDTA [Sigma], 2 mM EGTA [Sigma], 0.1 mM phenylmethylsulfonyl fluoride [Calbiochem], 10 ng/ml aprotinin [Sigma], 10 ng/ml leupeptin [ICN Biochemicals, Costa Mesa, CA], and 10 gm/ml pepstatin [ICN Biochemicals]) using a polytron as previously described (Florkiewicz *et al.*, 1995). Protein concentrations were determined using the BCA protein assay reagent (Pierce Chemical, Rockford, IL). Equal amounts of total protein from each sample were incubated with prewashed heparin-sepharose CL-6B (Pharmacia) for 2 h at 4°C. The heparin-binding fraction was analyzed by Western blot as previously described (Florkiewicz *et al.*, 1995) using a monoclonal antibody against human FGF-2 (Transduction Lab., Lexington, KY) followed by a rabbit anti-mouse secondary antibody (Jackson ImmunoResearch) and protein A-^{125}I. Signal was detected by autoradiography. A similar series of Western blots were carried out substituting polyclonal guinea pig FGF-2 antiserum for the monoclonal FGF-2 antibody.

RESULTS

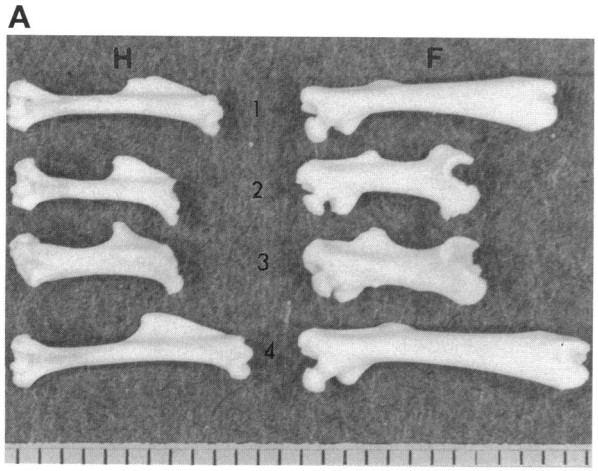
TgFGF214 Transgenic Mice

Genomic Southern blots detected the human FGF-2 transgene in 11 founder mice. Seven FGF-2 transgenic mice (TgFGF214) contained a low transgene copy number (less than three copies) and showed no overt phenotype whereas four high copy TgFGF214 mice appeared smaller with skeletal abnormalities. Two of the high copy founders failed to breed and died at 3–4 mo of age of unknown causes. Except for skeletal abnormalities, autopsy showed no lesions and no gross or microscopic morphological abnormalities as a cause of death. Three stable lines of FGF-2 transgenic mice, designated TgFGF214–15, TgFGF214–16, and TgFGF214–48, proved capable of breeding with germline transmission of the transgene. All three lines are now beyond F5 and consistently show a stable phenotype and transgene copy number.

Skeletal Alterations from FGF-2

Chondrodysplasia and macrocephaly were observed by gross examination of lines TgFGF214–15 and TgFGF214–48. Whole skeletons from TgFGF214–15 were further examined by Alizarin staining of the bones. A comparison of control and TgFGF214–15 skeletons of similar ages showed abnormalities in the vertebrae, ribs, long bones, and calvarium. The cervical vertebrae appeared compressed with surface irregularities and the thoracic vertebrae appeared enlarged and misshapen. Adjacent ribs were enlarged and flattened at the proximal and distal ends. The calvaria were enlarged over the occipital bones. The radius and ulna were shortened in the transgenic animals, the former showing an unusual angle of bending.

The long bones of the limbs in TgFGF214–15 mice were considerably shorter than those in the nontransgenic controls (Figure 1A). Precise measurements of long bone lengths between TgFGF214–15 mice and nontransgenic littermates show a 20–30% shortening of the ulna, radius, humerus, femur, and tibia in the TgFGF214–15 mice. All long bones were significantly shorter ($p < 0.001$) than nontransgenic littermate con-



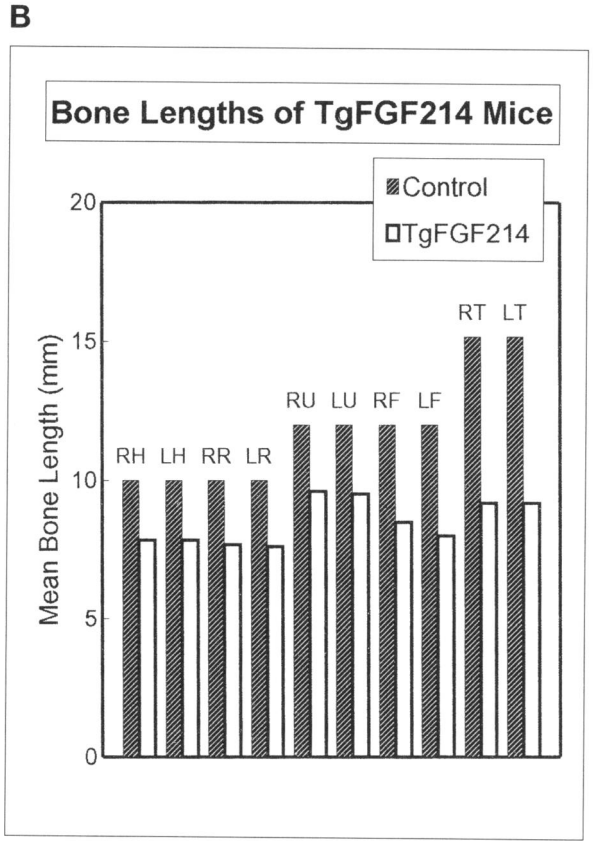


Figure 1. Chondrodysplasia is evident in TgFGF-214 mice. (A) Humeri (column H) and femurs (column F) from TgFGF214 mice are shorter than those from nontransgenic littermates. Shown are bones from a nontransgenic (row 1), a Tg FGF214–15 littermate (row 2), a TGFGF214–16 (row 3), and a nontransgenic littermate (row 4). TgFGF214 mice showed a noticeable chondrodysplasia in all three lines. Humeri and femurs shown are representative of results found in examination of the humerus, radius, ulna, femur, and tibia/fibula from TgFGF214–15 and -16 mice. A ruler with 1-mm graduations is included (bottom) for reference. (B) A graphical representation of data from measuring long bones shows chondrodysplasia in all TgFGF214–15 transgenic mice from a representative litter. Femurs, humeri, radii, ulnae, and tibias from transgenic mice all showed a 20–30% shortening compared with controls. Differences in bone lengths between the TgFGF214–15 mice and their nontransgenic littermates was statistically significant by a two-tailed *t*-test at $p < 0.001$.

trols (Figure 1B), whereas mean body weights of 20.4 gm for control and 19.0 gm for TgFGF214–15 mice were not significantly different. Normalization of the bone lengths with body weight shows continued significant differences between transgenic and control mice for bone length per gram body weight. Furthermore, comparison of TgFGF214–15 mice with nontransgenic littermates of similar body weight continues to show a reduction in the size of the long bones: a nontransgenic control mouse TgFGF214/15–48 weighed 19 grams with 10 mm humeri, 10 mm radii, 12 mm ulnae, 12 mm femurs, and 15 mm tibiae versus TgFGF214/15–45, and a transgenic littermate that weighed 20 grams had 8 mm humeri, 8 mm radii, 10 and 9 mm ulnae, 9 and 8 mm femurs, and 11 mm tibias. Thus the chondrodysplasia in TgFGF214–15 mice is not an artifact of gestational runting.

Histological and immunocytochemical analysis of femoral epiphyseal growth plates from adult TgFGF214–48 mice reveals chondrodysplasia (Figure 2). A thicker growth plate with chondrocyte hyperplasia and enhanced deposition and retention of extracellular matrix in the reserve zone is evident in TgFGF214 mice (Figure 2B). Normal growth plate chondrocytes (Figure 2A) contain cytoplasmic, nuclear, and extracellular FGF-2. They undergo rapid proliferation and matrix deposition, then differentiation, hypertrophy, and mineralization. In the differentiated zone (DZ) hypertrophic chondrocytes are FGF-2 immunonegative and FGF-2 localizes exclusively to the extracellular matrix (Figure 2A). Growth plates from TgFGF214 mice (Figure 2B) show a significant enlargement of the reserve zone (RZ) and proliferation zone (PZ) from chondrocyte hyperplasia and enhanced extracellular matrix deposition. The hypertrophic chondrocytes in the DZ are substantially diminished in the TgFGF214 mice and all chondrocytes within the entire growth plate contain high levels of cytoplasmic and nuclear FGF-2. In addition, the extracellular matrix surrounding the chondrocytes is also strongly stained for FGF-2 in TgFGF214–48 mice (Figure 2B). Osteoclasts and osteoblasts with normal morphologies were found but not counted in the TgFGF214–48 mice. Similar data were obtained for line TgFGF214–16. In these mice the chondrodysplasia and macrocephaly were not as severe, possibly because the transgene copy number was lower in TgFGF214–16 versus TgFGF214–15 and TgFGF214–48. Although the bones in line TgFGF214–48 were not measured, the mice appear to

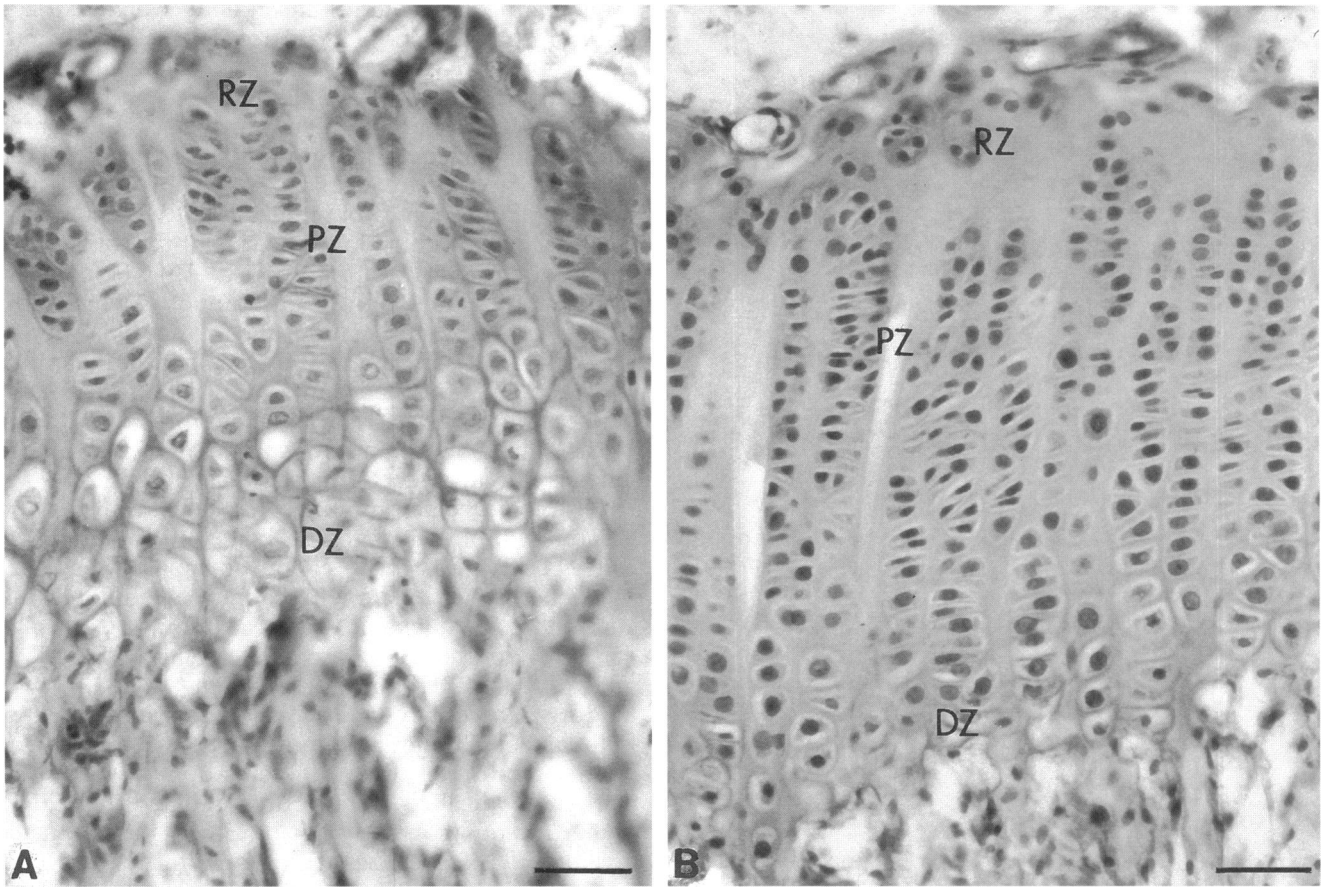


Figure 2. Comparative histology of epiphyseal growth plates from a TgFGF214–48 mouse and a nontransgenic littermate control shows chondrodysplasia from FGF-2 overexpression. (A) The control growth plate with a normal distribution of chondrocytes in the reserve zone (RZ), the proliferative zone (PZ), and hypertrophic chondrocytes in the differentiating zone (DZ). In the control growth plate staining for FGF-2 was evident in the nucleus, cytoplasm, and extracellular matrix and diminishes from the RZ to the DZ. The growth plate from a TgFGF214–48 mouse (B) is enlarged from chondrocyte hyperplasia and increased extracellular matrix in the RZ and the PZ, while hypertrophic chondrocytes are nearly absent in the diminished DZ. FGF-2 staining is abundant in all the zones of the TgFGF214 growth plate with a noticeable nuclear accumulation. (Bar, 15 μm).

have a similar phenotype to TgFGF214–15 with macrocephaly and chondrodysplasia. The common appearance of this effect in all TgFGF214 mice suggests that the chondrodysplasia is a direct result of the human FGF-2 transgene.

Measurement of body weights over time produced longitudinal growth curves for each strain (Figure 3). TgFGF214–16 and TgFGF214–48 showed no significant differences in body weight from 3 days postpartum to 160 days. Both sexes in the TgFGF214–15 mice showed a displacement of weight gain during growth from weaning until 50 days of age (Figure 3). The TgFGF214–15 female mice remained significantly smaller than their nontransgenic littermates throughout the course of the experiment (Figure 3A). In contrast to the females, at 50 days of age the TgFGF214–15 males achieved body weights similar to their nontransgenic littermates (Figure 3B).

Translational Regulation of FGF-2

Western blots were conducted to detect synthesis of the four human FGF-2 isoforms among brain, heart, lung, liver, kidney, spleen, and skeletal muscle from TgFGF214 mice. We relied on human and rodent FGF-2 high molecular weight isoforms migrating at different sizes in SDS-PAGE to distinguish human transgenic and rodent nontransgenic FGF-2. Control extracts from COS-1 cells transfected with human (Figure 4, lane CH) and rodent (Figure 4, lane CR) FGF-2 expression vectors show that the human isoforms (22, 23, and 24 kDa) are larger than the rodent species (20 and 21 kDa). Figure 4 also shows that the 22- and 23-kDa human isoforms do not separate well by 12% SDS-PAGE and are generally detected as a single, broad band. Both the human and rodent produce FGF-2 that runs at 18 kDa. Thus an increase in

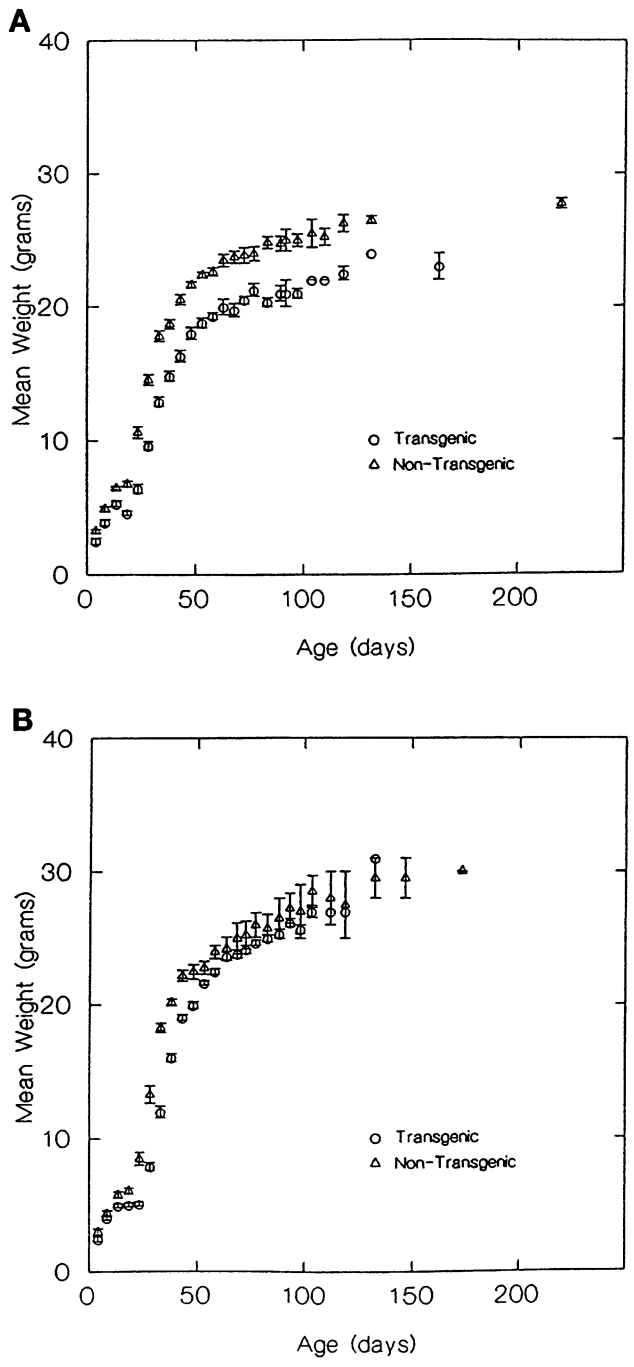


Figure 3. Longitudinal growth curves for TgFGF214 transgenic mice. All three lines of TgFGF214 male mice were bred under similar conditions to nontransgenic female littermates. Transgenic and nontransgenic offspring were weighed every day for the first 60 days, then weekly thereafter up to 250 days. No significant differences in body weight were evident in the TgFGF214–16 and the TgFGF214–48 strains. Both sexes in the TgFGF214–15 strain showed diminished mean weight gain from 20–50 days (A and B). This trend remained constant in TgFGF214/15 females that never achieved body weight of their nontransgenic littermates (A). Transgenic males, however, recovered to weights similar to their nontransgenic littermates (B).

the signal strength of the 18-kDa band between the transgenic and nontransgenic mice suggests, but does not prove that the transgene is overexpressing 18-kDa human FGF-2. These results are consistent with previously published data (Florkiewicz and Sommer, 1989).

The Western blots show that brain, heart, lung, liver, and spleen from TgFGF214 mice each contain human 24- and 23/22-kDa immunoreactive bands and a quantitative increase in the 18-kDa signal (Figure 4). Careful examination of the FGF-2 isoforms in these transgenic samples and comparison with controls and nontransgenic littermates shows expression of human FGF-2 (18, 22, 23, and 24 kDa) over the rodent background (18, 20, and 21 kDa). Skeletal muscle and kidney extracts from both TgFGF214 and nontransgenic mice were unique, where only an 18-kDa signal was detected and neither the human or rodent FGF-2 high molecular weight proteins are found (Figure 4). However, a quantitative increase in 18-kDa FGF-2 is found in the transgenic extracts over the nontransgenic samples for both kidney and skeletal muscle. A band at approximately 23 kDa in skeletal muscle (Figure 4) of both TgFGF214 and nontransgenic mice is believed to be artifact. To assess this, the Western blots were repeated in the same manner, but guinea pig antisera to FGF-2 was substituted for the FGF-2 monoclonal antibody. The banding patterns were the same with the guinea pig antisera, but the artifact (23-kDa band) disappeared (not shown). In general, comparison of FGF-2 expression among the organs and tissues examined revealed that the molar ratios of the high molecular weight FGF-2 protein isoforms differ between tissues (or organs). This result is true for samples derived from either a transgenic or a nontransgenic mouse. These data suggest that the FGF-2 human transgenic mRNA is translationally regulated similarly to the endogenous murine FGF-2 mRNA, in a tissue-specific manner.

RNase protection was conducted on liver and kidney samples from TgFGF214 and nontransgenic control mice to confirm the presence of the alternative translation start sites in the transgenic human FGF-2 mRNA. Liver and kidney were chosen as representative samples because liver expresses all four human FGF-2 isoforms while kidney expresses only 18-kDa FGF-2 in the TgFGF214 mice (Figure 4), suggesting tissue-specific translational regulation from the human FGF-2 transgene. A probe was designed that is specific to an upstream segment of the human CUG transgene to protect the region containing the alternative human translation start sites. A similar region is not present in murine FGF-2 mRNA, thus there is no chance of cross-hybridization of the probe. RNase protection showed specific expression of the human transgene in liver and kidney from the TgFGF214 mice (Figure 5) while the protected region was absent from

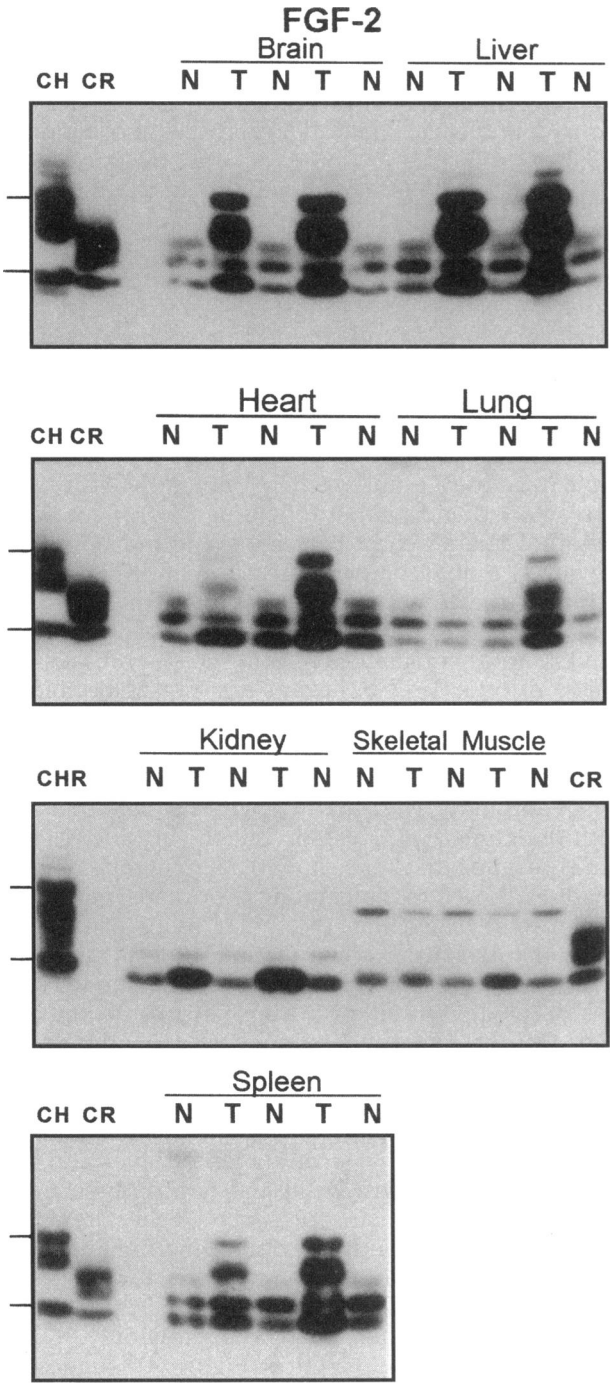


Figure 4. FGF-2 protein isoform expression is translationally regulated in a tissue-specific manner. Autoradiographs of Western blots from organs and tissues of FGF-2 transgenic mice and their nontransgenic littermates are shown. Brain, liver, heart, lung, kidney, spleen, and skeletal muscle were all examined. Transgenic (T) brain, liver, heart, spleen, and lung all show expression of human FGF-2 isoforms (24, 23, 22, and 18 kDa) when compared with the background (21, 20, and 18 kDa) of the nontransgenic (N) mice. Kidney and skeletal muscle show predominant expression of 18-kDa FGF-2 in both transgenic and nontransgenic mice. Although the transgenic mice generally express more FGF-2 protein in kidney and their nontransgenic littermates. The RNase protection assays also showed that the transgenic mRNA contained the codons necessary for translation of the high molecular weight human FGF-2 isoforms. Consideration of Western blot and RNase protection results show that human transgenic mRNA in both liver and kidney contain all the necessary codons to produce all FGF-2 protein isoforms but the liver makes 22-, 23-, and 24-kDa FGF-2 while the kidney specifically makes the 18-kDa isoform. This suggests that these organs are capable of selective translational regulation.

RT-PCR was conducted to confirm expression of human FGF-2 in tissues and organs (Figure 6). Consistently higher levels of FGF-2 expression were evident by RT-PCR in all organs and tissues examined from TgFGF214 mouse. Semi-quantitative analysis with actin controls (Figure 6) showed elevated levels of FGF-2 mRNA in brain, heart, lung, liver, kidney, spleen, and skeletal muscle from TgFGF-214 mice versus nontransgenic controls. It should be noted that the primers used in this reaction were not specific to human FGF-2, thus endogenous murine FGF-2 may have been amplified along with the transgenic human FGF-2. In addition, RT-PCR with a lead primer in the intron and a second primer in the FGF-2 coding sequence failed to yield a product (not shown), suggesting that the intron is not encoded in the transgenic mRNA. This provides further evidence that the mRNA was spliced between the intron and the most upstream (24 kDa) CUG codon.

Smooth Muscle Hypertrophy

Because FGF-2 has been shown to alter growth and differentiation of vascular smooth muscle (Lindner *et al.*, 1993), the effects of the FGF-2 transgene were examined by an in vitro analysis of smooth muscle cells from TgFGF214 mice and nontransgenic littermates. Although the transgenic-derived VSMC expressed approximately fivefold greater FGF-2 than cells from nontransgenic animals, both FGF-2–expressing and control cells underwent growth arrest upon withdrawal of serum from the culture medium (not shown). Comparison of the cellular DNA and protein

Figure 4 (cont). skeletal muscle, human 18-kDa FGF-2 cannot be distinguished from murine 18-kDa FGF-2. Differences in the molar ratios of FGF-2 isoforms are evident when the lung is compared with the heart or when the kidney is compared with the liver or brain. This suggests that individual organs or tissues are capable of selective expression from the alternative translation start sites in both the transgene and in the endogenous murine FGF-2 gene. The transgene appears to be regulated in the same manner as the endogenous murine FGF-2 gene in all the samples. The heparin binding fraction from COS cells transfected with the human or rodent FGF-2 cDNA is included as a reference. Marks to the left of each photo mark the 18 (lower)- and 24 (upper)-kDa FGF-2 isoforms. CH, Cos human; CR, Cos rodent; CHR, Cos human mixed with Cos rodent.

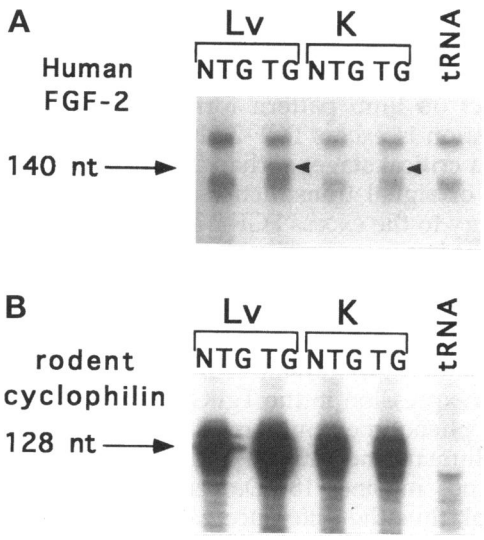


Figure 5. The coding sequences for FGF-2 high molecular weight isoforms are present in the transgenic mRNA. RNase Protection assays show transcripts of 140 nt in liver (Lv) and kidney (K) from the transgene in TgFGF214 mice that are absent in nontransgenic littermates. The protected fragment is approximately 80 bp shorter than predicted, suggesting that the message was spliced just upstream of the CUG codon for the 24-kDa FGF-2 isoform in the transgene. This result is confirmed by Western blots that show a 24-kDa band (Figure 4). (B) Rodent cyclophilin was used as a control.

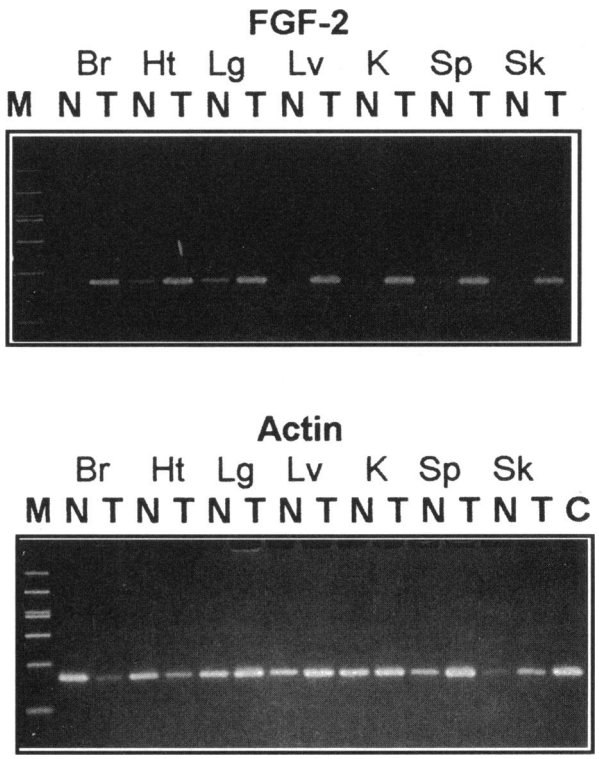


Figure 6. RT-PCR shows elevated levels of FGF-2 message in TgFGF214 mice (T) versus their nontransgenic (N) littermates. RT-PCR was conducted with primers corresponding to the second and third exons of the FGF-2 gene. The primers are neither human nor murine specific, thus the product reflects total FGF-2 transcription in brain (Br), heart (Ht), lung (Lg), liver (Lv), kidney (K), spleen (Sp), and skeletal muscle (Sk). FGF-2 mRNA is present or elevated in all transgenic mice (upper panel). Only heart, lung, and spleen showed any endogenous FGF-2 mRNA, while the transgene was found at high levels in all tissues and organs tested. β-Actin was used as a control (lower panel). Markers (M) shown are (from top) 1000, 700, 525, 500, 400, 300, and 200 bp. The "C" control (C) in the lower panel represents a β-actin–amplified positive control from bovine brain.

content of transgenic and control cells demonstrated similar amounts of DNA, but increased protein content was found in the transgenic cells (Figure 7). When corrected for DNA content, transgenic cells had 66% more protein than controls, indicating that quiescent FGF-2–overexpressing cells exhibit a state of basal hypertrophy relative to control cells. These results were identical if protein content was indexed to cell number rather than DNA content (not shown). No overt phenotype, in the form of hemangioma or microangiopathy, was observed in the endothelium. There were no signs of aberrant neovascularization in any of the offspring observed at any age up to 18 mo. In addition, TgFGF214 mice aged up to 2 yr showed no sign of increased cancer rates.

DISCUSSION

Abnormalities in the skeletal system were found in all the TgFGF214 mice. The most severe abnormalities appeared in the high copy founder mice TgFGF214–19, -42 and - 30 that died prematurely. This phenotype also appeared in the three surviving lines TgFGF214–15, -16, and -48 to varying degrees that appear to correlate with the dose of the transgene. The TgFGF214–15 and TgFGF214–48 mice have high copy numbers and severe chondrodysplasia while the TgFGF214–16 mice have a low copy number and mild chondrodysplasia. Morphological examination of the growth plates from these mice demonstrates a phenotype directly related to FGF-2 overexpression associated with chondrocyte growth and differentiation (Figure 2). The growth curves for the three lines (Figure 3) also suggest that the chondrodysplasia is a phenotype resulting from a direct effect of FGF-2 on bone versus a general runting phenomenon. The chondrodysplasia resulting from overexpression of the FGF-2 ligand is interesting in relation to skeletal malformations in human syndromes associated with mutations in the FGFRs. Mutation of FGFR1 causes Pfeiffer syndrome (Muenke *et al.*, 1994), mutation of FGFR-2 causes Jackson-Weiss and Crouzon syndromes (Jabs *et al.*, 1994; Reardon *et al.*, 1994), and mutation of FGFR-3 causes achondroplasia (Rousseau *et al.*, 1994; Shiang *et al.*, 1994) and thanatophoric dys-

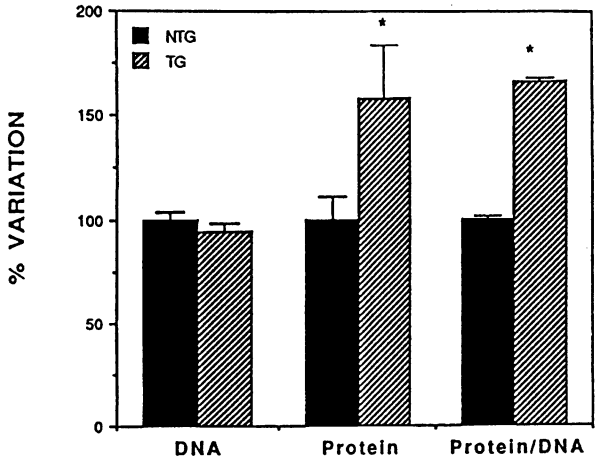


Figure 7. VSMC in TgFGF214 exhibit hypertrophy. Aortae from TgFGF214 mice and their nontransgenic littermates were explanted on collagen for isolation of VSMC. DNA and protein content were measured in serum-starved growth arrested cells. DNA levels were not significantly different between TgFGF214 mice and nontransgenic controls. Protein levels were significantly higher in the TgFGF214 VSMC producing a significantly higher protein/DNA ratio indicative of VSMC hypertrophy.

plasia (Tavormina *et al.*, 1995). Bone is a primary target tissue of all of the human dysmorphic syndromes from FGFR mutations, and bone is primarily affected in the phenotype of the TgFGF214 mice, suggesting a key role for FGF-2 in bone morphogenesis. Overexpression of the FGF-2 ligand causing chondrodysplasia suggests that the phenotypes in the human FGFR syndromes may result from aberrant, constitutive activation of the receptors. Because these FGFRs are cell surface tyrosine kinases, the latter phenomenon may also involve aberrant signal transduction. Chondrodysplasia and macrocephaly in FGF-2 transgenic mice suggests that it may be possible to produce mouse models for the FGFR-related human syndromes.

We found no evidence of missing or fused digits in limbs from TgFGF214 mice as described in the literature where excess exogenous FGF-2 results in developmental abnormalities in limb patterns (Olwin *et al.*, 1994). There are several possible explanations for this discrepancy. The phosphoglycerate kinase promoter was chosen because it is presumed to express in all tissues through all stages of development. However, our data are incomplete regarding the specific temporal and spatial expression patterns for the human FGF-2 transgene. Although the litters are smaller for the TgFGF214 mice versus nontransgenic mice there is not an excess of resorption plaques in the uterus, suggesting that implanted embryos generally develop to term. Moreover, the ratios of transgenic to nontransgenic littermates are 1:1 for hemizygous to nontransgenic breedings, suggesting no loss of transgenic mice in either pre-implantation or post-implantation stages of development. It is possible that the human FGF-2 transgene may not be expressed in the appropriate cells at the correct stage of development to have an effect on limb pattern formation. An alternative explanation is excess FGF-2 altering developing cells before a critical stage, perhaps by down regulation of FGFRs or signal transduction genes, making tissues refractory to the excess FGF-2 and suppressing a phenotype at later stages. The biological effects may differ between adding exogenous FGF-2 to a culture system as in the limb pattern defect studies, versus endogenous overexpression of FGF-2 as in the TgFGF214 mice. Finally, the human coding sequence was used for overexpression in the TgFGF214 mice and the observed phenotype could result from differences between human and murine nuclear isoforms. The human and murine 18-kDa isoforms are virtually identical, thus the difference would have to lie in the nuclear effects. This phenomenon would be interesting in regard to the human chondrodysplasias mapped to FGFRs because it suggests that they can be recapitulated in the mouse by overexpressing the human nuclear isoforms of FGF-2.

The Western blot data shown here (Figure 4) suggest that endogenous murine FGF-2 mRNA and human FGF-2 mRNA produced from the transgene are translationally regulated in a similar manner. The human isoforms can all arise from a single mRNA (Florkiewicz and Sommer, 1989) by amino terminal extension of the 18-kDa isoform from CUG codons, and the rodent mRNA appears to share this characteristic, including predicted mRNA secondary structure (Florkiewicz, unpublished data). In spite of quantitatively more mRNA in transgenic mice, the isoform profiles for each tissue or organ remain qualitatively similar for human and murine FGF-2. These data suggest that murine tissues do not distinguish between endogenous mRNA and human mRNA derived from the transgene, allowing translation initiation to start from the CUG codons in either transgenic or endogenous mRNA to produce both human and murine FGF-2 isoforms. Thus the tissue-specific regulatory mechanisms are conserved between species and the TgFGF214 transgenic mice may provide an excellent system to study translational regulation of FGF-2 expression. This may be particularly important for diseases contingent upon FGF-2, where qualitative changes in FGF-2 isoform expression or alteration of isoform ratios in cells may be equally as important as quantitative changes in FGF-2 expression.

Phenotypes may exist in the TgFGF214 mice that are not as obvious as the skeletal malformations described here. The smooth muscle data show that transgenic overexpression of FGF-2 results in hypertrophied VSMC but is not sufficient to permit unrestrained vascular smooth muscle growth after withdrawal of serum from the medium. This suggests that FGF-2 has

a paracrine role in VSMC development and that intracellular FGF-2 does not stimulate smooth muscle cell growth. Thus FGF-2 may need to bind a putative tyrosine kinase cell surface receptor to stimulate VSMC growth. Further studies will focus on the relationship of exogenous FGF-2 and its relationship or dependency on cell surface receptors on VSMCs. Because FGF-2 is also a potent angiogenic factor, we expected that TgFGF214 mice might have overt vascular abnormalities, such as hemangioma, or be overly susceptible to tumor formation because of enhanced tumor angiogenesis. In addition, some reports suggest that FGF-2 itself may be neoplastic (Rifkin and Moscatelli, 1989; Huang and Wright, 1994). The lack of increased tumor frequency in the TgFGF214 mice suggests that FGF-2 is not neoplastic. However, preliminary experiments in our lab (Coffin, unpublished data) where we have induced tumor formation in TgFGF214 mice and their nontransgenic littermates suggest that the FGF-2–overexpression mice are susceptible to increased tumor growth and metastasis. This susceptibility to malignancy correlates with increased tumor angiogenesis, suggesting that FGF-2 may not be neoplastic and primarily functions as an angiogenic factor.

The most interesting results from generating FGF-2 transgenic mice are the effects of excess FGF-2 on the skeletal system and the isoform-specific tissue-dependent expression patterns for FGF-2 proteins. We have identified bone as the principle target tissue for the physiological effects of FGF-2 overexpression during growth and development. This phenotype is consistent with in vitro data showing changes in the growth and differentiation of chondrocytes by addition of exogenous FGF-2 (Trippel *et al.*, 1993) and human syndromes where mutation of FGFR tyrosine kinases primarily affect the skeletal system (Jabs *et al.*, 1994; Muenke *et al.*, 1994; Reardon *et al.*, 1994; Rousseau *et al.*, 1994; Shiang *et al.*, 1994; Tavormina *et al.*, 1995). The Western blots and RNase protection assays suggest that tissue-specific translational regulation is a means for differentially regulating expression of FGF-2 nuclear and cytoplasmic protein isoforms. The functional significance of multiple FGF-2 isoforms, translationally regulated by initiation from CUG and AUG codons, is difficult to investigate. Translational assays with human mRNA and wheat germ extract yield results consistent with the data shown here (Florkiewicz *et al.*, 1991b). The TgFGF-214 mice are the first in a series of experiments to determine whether nuclear and cytoplasmic/extracellular FGF-2 have different biological effects. We are now producing transgenic mice that specifically overexpress either nuclear or cytoplasmic FGF-2 to determine whether the phenotypes observed here are isoform specific.

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