The AP1 transcription factor Fra2 is required for efficient cartilage development

Florian Karreth, Astrid Hoebertz, Harald Scheuch, Robert Eferl and Erwin F. Wagner*

Research Institute of Molecular Pathology (I.M.P.), Dr Bohr-Gasse 7, 1030 Vienna, Austria
*Author for correspondence (e-mail: wagner@imp.univie.ac.at)

Accepted 20 August 2004

Development 131, 5717-5725
Published by The Company of Biologists 2004
doi:10.1242/dev.01414

Summary

The Fos-related AP1 transcription factor Fra2 (encoded by *Fosl2*) is expressed in various epithelial cells as well as in cartilaginous structures. We studied the role of Fra2 in cartilage development. The absence of Fra2 in embryos and newborns leads to reduced zones of hypertrophic chondrocytes and impaired matrix deposition in femoral and tibial growth plates, probably owing to impaired differentiation into hypertrophic chondrocytes. In addition, hypertrophic differentiation and ossification of primordial arches of the developing vertebrae are delayed in Fra2-deficient embryos. Primary *Fosl2*$^{-/-}$ chondrocytes exhibit decreased hypertrophic differentiation and remain in a proliferative state longer than wild-type cells. As pups lacking Fra2 die shortly after birth, we generated mice carrying 'floxed' *Fosl2* alleles and crossed them to coll2a1-Cre mice, allowing investigation of postnatal cartilage development. The coll2a1-Cre, *Fosl2*$^{f/f}$ mice die between 10 and 25 days after birth, are growth retarded and display smaller growth plates similar to *Fosl2*$^{-/-}$ embryos. In addition, these mice suffer from a kyphosis-like phenotype, an abnormal bending of the spine. Hence, Fra2 is a novel transcription factor important for skeletogenesis by affecting chondrocyte differentiation.

Key words: AP1, AP-1, Fra2, Fra-2, Growth plate, Cartilage, Type X collagen, Mouse

Introduction

The vertebrate skeleton is composed of cartilage and bone. Cartilage controls longitudinal bone growth, serves as scaffold for bone formation and gives flexibility to the skeleton by its presence around joints. The mesenchymal precursors form aggregates at embryonic day 10 (E10) in the developing limbs, thereby initiating endochondral ossification by differentiating into chondrocytes. Chondrocytes first proliferate, then differentiate into prehypertrophic chondrocytes and eventually become hypertrophic chondrocytes, thus forming the typical layer-structured subpopulations of the growth plate (Karsenty and Wagner, 2002). The life cycle of chondrocytes from proliferation to hypertrophy is controlled by a variety of factors expressed in chondrocytes and the surrounding perichondrium, such as Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) (Kobayashi et al., 2002; St-Jacques et al., 1999), fibroblast growth factor (FGF) signaling (Ohbayashi et al., 2002; Liu et al., 2002; Deng et al., 1996) and the runt family transcription factor Runx2 (Komori, 2002). Hypertrophic chondrocytes produce a type X collagen-rich extracellular matrix (ECM) before they die through apoptosis. This ECM stimulates blood vessel invasion, which attracts osteoblasts and osteoclasts, the two bone-specific cell types. Osteoblasts share a mesenchymal precursor with chondrocytes (Ducy et al., 1997) and use the cartilage ECM as scaffold to eventually replace it with a type I collagen-rich bone matrix. Osteoclasts derive from the macrophage/monocyte lineage, and resorb cartilage and the bone ECM. For healthy bone development, an equilibrium between osteoblastic and osteoclastic activity has to be maintained and a shift towards one side results in either reduced (osteopenia/osteoporosis) or increased (osteopetrosis/osteosclerosis) bone mass (Karsenty, 1999). Furthermore, cartilage defects can cause chondrodysplasias in mice and humans (Cohen, 2002; Li and Olsen, 1997).

The AP1 (activator protein 1) transcription factor consists of dimers of the Fos (Fos, Fra1, Fra2 and FosB) and Jun (Jun, JunB and JunD) families of basic leucine zipper domain proteins. AP1 is involved in several biological processes, including differentiation, proliferation, apoptosis and oncogenic transformation (Jochum et al., 2001). Jun proteins seem to play important roles during development as the absence of Jun (Hilberg et al., 1993; Johnson et al., 1993) and JunB (Schorpp-Kistner et al., 1999) results in embryonic lethality. A bone phenotype was described only recently in embryo-specific *Junb* knockout mice, which develop osteopenia and suffer from a chronic myeloid leukemia (CML)-like disease (Hess et al., 2003; Kenner et al., 2004), as well as in cartilage-specific *Jun* knockout mice, which display scoliosis (Behrens et al., 2003). All four members of the Fos family seem to be involved in bone development. *Fos* knockout mice lack osteoclasts because of a complete block in osteoclast differentiation, resulting in an osteopetrotic phenotype (Wang et al., 1992; Grigoriadis et al., 1994), whereas ubiquitous expression of Fos in transgenic mice leads to the formation of osteosarcomas (Ruther et al., 1989). When Fra1 (encoded by *Fosl1*) is expressed from the *Fos* locus, the osteopetrotic *Fos* knockout phenotype is partly rescued (Fleischmann et al., 2000). Overexpression of ΔFosB, a splice variant of FosB,

leads to an osteosclerotic phenotype because of increased numbers of mature osteoblasts (Sabatakos et al., 2000). Similarly, transgenic mice overexpressing Fra1 develop osteosclerosis because of a cell-autonomous increase in the number of mature osteoblasts (Jochum et al., 2000), whereas embryo-specific *Fosl1* knockout mice display osteopenia (Eferl et al., 2004). Little is known about the role of Fos proteins, in particular Fra2 (encoded by *Fosl2*), in cartilage development. Overexpression of Fos in embryonic stem (ES) cell chimeras leads to the development of chondrosarcomas (Wang et al., 1991). By contrast, overexpression of Fos in the chondrogenic cell line ATDC5 inhibits chondrocyte differentiation (Thomas et al., 2000). Fra2 is expressed at high levels in ovary, stomach, intestine, brain, lung and heart (Foletta et al., 1994), and in differentiating epithelia, the central nervous system and developing cartilage (Carrasco and Bravo, 1995). In addition, expression of Fra2 has been found to be distinct from other Fos members, suggesting that it has unique functions during embryonic development and adulthood.

As Fra2 is expressed during bone development, in particular in differentiating chondrocytes (Carrasco and Bravo, 1995), we determined the role of Fra2 in cartilage biology and bone growth. Initially, we investigated its function in embryos and newborns lacking Fra2. The zones of hypertrophic chondrocytes were narrower throughout embryonic and early postnatal development and less calcified matrix was deposited in *Fosl2*$^{-/-}$ growth plates. This is probably due to impaired chondrocyte differentiation in vivo and in vitro. Moreover, endochondral ossification was delayed in developing vertebral columns of *Fosl2*$^{-/-}$ embryos. As *Fosl2*$^{-/-}$ pups die shortly after birth, we generated floxed *Fosl2* mice and crossed them to coll2a1-Cre transgenic mice. The conditional *Fosl2* knockout mice have a rather broad spectrum of *Fosl2* deletion; however, they display a similar defect in chondrocyte differentiation and suffer from a kyphosis-like phenotype.

Materials and methods

Animals and tissue fixation

The generation of *Fosl2*$^{-/-}$ mice and mice carrying floxed *Fosl2* (*Fosl2*$^{f/f}$) alleles will be described elsewhere (R.E., unpublished). Mice carrying floxed *Fosl2* alleles were crossed to coll2a1-Cre mice (Haigh et al., 2000). The genetic background was C57Bl/6×129 for the *Fosl2* knockout mice and 129×B6CBAF1 for the coll2a1-Cre; *Fosl2*$^{fl/fl}$ mice. Genotypes of each animal were determined by PCR analysis with tail DNA as template. Primers used for genotyping of *Fosl2*$^{-/-}$ mice are available upon request. Embryos were isolated at the appropriate time points by Caesarian section. Embryos and limbs of newborn mice were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Tissues were dehydrated and embedded in paraffin wax. Sections were cut (5 μm) and used for experiments.

Skeletal staining

Animals were skinned, eviscerated and dehydrated in 95% ethanol overnight and in acetone again overnight. Skeletons were stained with 0.015% Alcian Blue, 0.05% Alizarin Red and 5% acetic acid in 70% ethanol for several days. Next, the skeletons were cleared in 1% KOH for an age dependent period, passed through a decreasing KOH series and stored in glycerol.

In situ hybridization

Digoxigenin-labeled riboprobes were synthesized according to the manufacturer's instructions (DIG RNA labeling kit, Boehringer-Mannheim). For in situ hybridization analyses, sections were deparaffinized and hybridization was performed according to standard procedures (Murtaugh et al., 1999). The signal was detected according to the manufacturer's (Boehringer-Mannheim) instructions using BM-purple AP-substrate solution. Sections were then washed, fixed in 4% PFA and mounted.

BrdU labeling

For in vivo labeling, 100 μg/g body weight BrdU were injected intraperitoneally in pregnant females at E17.5 and E18.5, and embryos were isolated by Caesarian section 32 hours and 2 hours later, respectively. Sections were deparaffinized, unmasked by boiling in citrate buffer (0.1 mM citrate acid, 0.8 mM sodium citrate), blocked in 20% horse serum and incubated with a FITC-labeled α-BrdU antibody (Becton Dickinson) for 30 minutes in the dark. Cells were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI). At least 200 cells were counted in the zone of proliferating chondrocytes.

Primary rib cage chondrocytes were incubated with 4 μM/ml BrdU for 2 hours. Cells were fixed in 70% ethanol and permeabilized with 0.07 N NaOH for 2 minutes at room temperature. Cells were incubated with FITC-labeled α-BrdU antibody for 30 minutes in the dark, counterstained with DAPI and mounted.

Von Kossa staining

Paraffin sections were deparaffinized and incubated in 2% silver nitrate in a coplin jar placed directly in front of a 60 W lamp for 1 hour to detect matrix-bound Ca^{2+}. After the staining, sections were fixed with 2.5% sodium thiosulfate for 5 minutes. The sections were washed, dehydrated and mounted.

Ki67 staining

Paraffin embedded sections were deparaffinized and unmasked as described above. To block endogenous peroxidase activity, sections were incubated in 3% H_2O_2 for 30 minutes at room temperature. Unspecific binding sites were blocked using 20% horse serum for 20 minutes at room temperature, followed by incubation with an α-Ki67 antibody (Novo Castra) for 1 hour at room temperature. Secondary α-rabbit antibody and Vectastain solution (Vectastain ABC kit, Vector Laboratories) were used according to the manufacturer's recommendations. After washing, the sections were incubated for 2-10 minutes with DAB substrate solution (DAB Peroxidase Substrate kit, Vector Laboratories), washed and mounted.

Cell culture

Rib cage chondrocytes were isolated from *Fosl2*$^{-/-}$ and *Fosl2* wild-type neonatal mice. Rib cages were sequentially digested twice for 30 minutes and once for 4 hours at 37°C in a 0.2% collagenase solution in serum-free Dulbecco's Modified Eagle Medium (DMEM) with antibiotics (100 μg/ml Streptomycin, 100 U/ml Penicillin). Single cells were cultured overnight over 1.5% agarose in DMEM, 10% fetal calf serum (FCS), 100 μg/ml Streptomycin, 100 U/ml Penicillin and 5 μM L-Glutamate (P/S/G) to obtain fibroblast-free cultures.

Cumulative cell number assay

Primary rib cage chondrocytes were seeded in six-well plates at a number of 1×10^5 cells/well. After 2 days in culture, cells were counted and replated at 1×10^5 cells per well. Counting was repeated for at least four passages.

Differentiation assay

Primary chondrocytes were seeded in 24-well plates ($1\text{-}2\times10^5$ cells/well) and cultured in DMEM, 10% FCS, P/S/G, 5 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 12 days. Half of the media was exchanged every other day. Differentiated cells were stained in 0.1% Alcian Blue in 0.1 N HCl. The dye was extracted with 4M guanidine-hydrochloride and absorbance measured at 595 nm.

Semi-quantitative RT-PCR and real time PCR

Total RNA was isolated from primary rib cage chondrocytes or knee joints from newborn mice (P2) using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. RNA (2-4 μg) was used for cDNA synthesis using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) and 1 μl of Random Primers (Invitrogen) according to the manufacturer's instructions. After an initial denaturation at 95°C for 2 minutes, the PCR reactions were carried out as follows: denaturing for 30 seconds at 95°C, annealing for 45 seconds at 55°C and extension for 90 seconds at 65°C. The reaction was completed by a 7 minute extension step at 65°C. For real-time PCR, light cycler Fast start DNA Master SYBR Green (Roche Diagnostics) was used. The following primers for were used: aggrecan (forward) 5′-tcgcccaggctccaccagatact-3′ and (reverse) 5′-ccagccagccagcatagcacttgt-3′; type II collagen (forward) 5′-gcgagaggggactgaagggacacc-3′ and (reverse) 5′-cggggctgcggatgctctcaat-3′; type X collagen (forward) 5′-gacccccctggcccctctgga-3′ and (reverse) 5′-atctcaccttttagcgcctggaatg-3′; Ihh (forward) 5′-caagcagttcagcccc-caacg-3′ and (reverse) 5′-acgtgggc-cttggactcgta-3′; Fosl2 (forward) 5′-ttatcccgggaactttgacacctc-3′ and (reverse) 5′-cggcgttcctcggggctgatt-3′; tubulin (forward) 5′-gacagagccaaact-gagcacc-3′ and (reverse) 5′-caacgtcaa-gacggccgtgtg-3′. The expression levels of RNA transcripts were calculated with the comparative CT method. The individual RNA levels were normalized for tubulin and depicted as relative expression levels with the corresponding controls set to 1.

RNase protection assay (RPA)

Total RNA was isolated with the TRIzol protocol (Sigma) and 10 μg were used for each RPA reaction. RPA was performed using the RiboQuant multiprobe RNase protection assay system mJun/Fos (PharMingen) according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times and carried out in triplicate. Statistical analysis was performed using Student's *t*-test, $P<0.05$ was accepted as significant. Data are shown as mean and the error bars represent the standard deviation.

Results

Reduced zone of hypertrophic chondrocytes in embryos and newborns lacking Fra2

To gain insights into the role of Fra2 in developing cartilage,

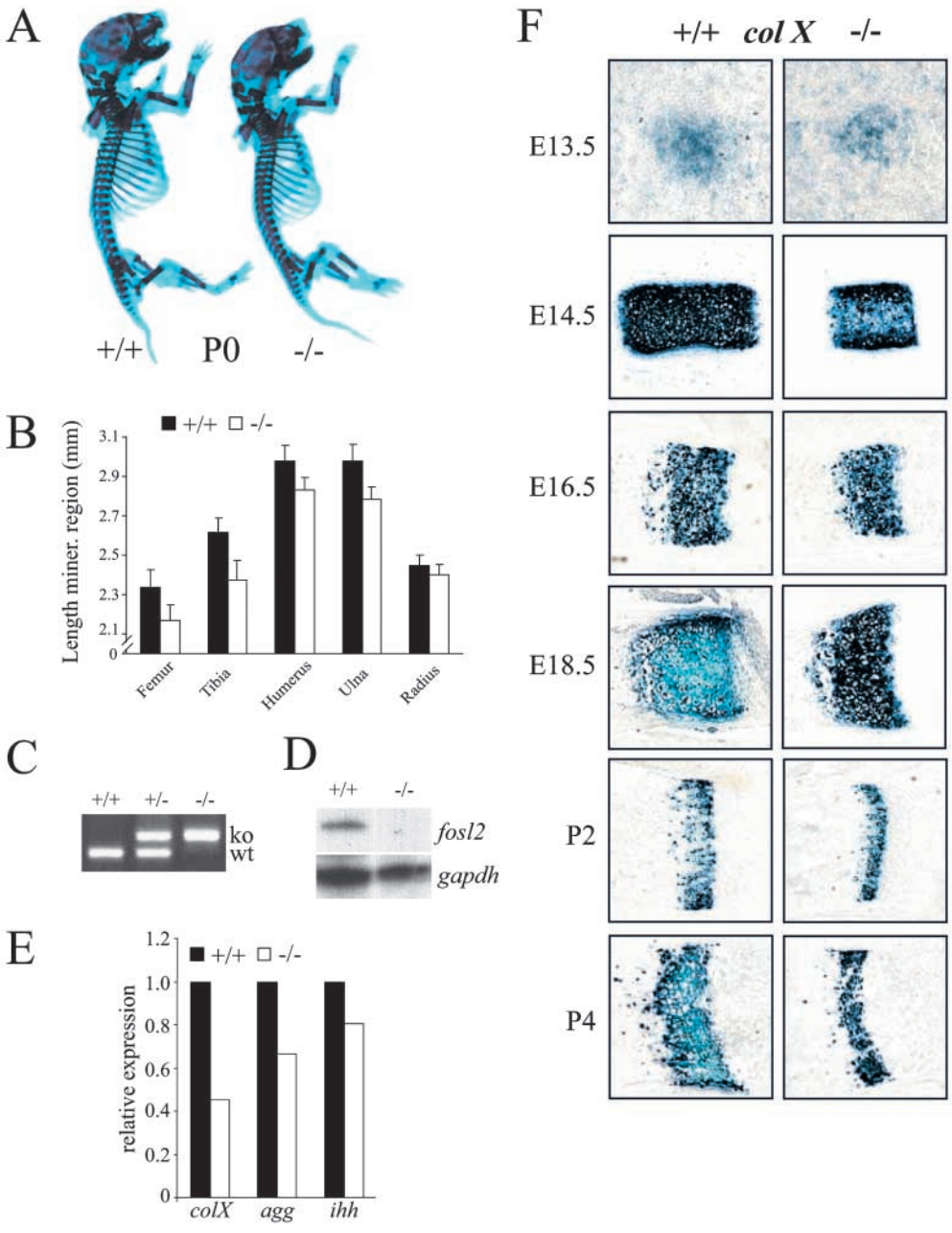

Fig. 1. Reduced zones of hypertrophic chondrocytes in *Fosl2*$^{-/-}$ embryos and newborn mice. (A) Skeletal staining of a *Fosl2*$^{-/-}$ newborn and wild-type littermate at P0. (B) Length of mineralized regions of knock-out and wild-type littermates at P0; bars represent mean value±s.e.m.; $n=4$. (C) Genotyping-PCR of DNA from wild-type, heterozygous and *Fosl2*-null newborn mice. (D) Expression of *Fosl2* in primary rib cage chondrocytes and its absence in mutant cells. Primary chondrocytes were cultured for 3 days prior to RNA isolation and RNase protection assay. GAPDH was used as loading control. (E) Real-time PCR of cartilage markers. Relative expression of type X collagen (*colX*), aggrecan (*agg*) and *Ihh* is shown. Expression levels were normalized to tubulin expression. The mean of two independent measurements is shown. (F) In situ hybridization on sections of embryonic and postnatal femoral growth plates of *Fosl2*$^{-/-}$ mice and littermate controls using a type X collagen antisense probe as a marker for hypertrophic zones. Pictures were taken at 200× (E13.5), 100× (E14.5, E16.5, E18.5) and 50× (P2, P4) magnification.

where Fra2 is expressed, we investigated whether the function of growth plate chondrocytes as well as spine development was affected by the absence of Fra2. No gross skeletal malformations were observed by skeletal staining of *Fosl2*$^{-/-}$ E18.5 embryos and newborn mice, although the mineralized Alizarin Red-stained regions of the limbs were slightly, but not significantly, shorter in *Fosl2*$^{-/-}$ newborns compared with control littermates (Fig. 1A,B). The deletion of *Fosl2* was verified by PCR from tail DNA (Fig. 1C). Moreover, *Fosl2* was found to be expressed in rib cage chondrocytes and expression was not detectable in the knockout cells by RNase protection assay (Fig. 1D). The expression of the other AP1 members was unchanged in Fra2-deficient primary chondrocytes (data not shown). We next analyzed the expression of several cartilage marker genes in total RNA isolated from knee joints by real time PCR analysis. We observed reduced expression of the ECM proteins type X collagen and aggrecan and slightly reduced expression of *Ihh* (Fig. 1E).

To investigate the effect of Fra2 deficiency on the growth plate architecture, expression of several markers expressed in different chondrocyte subpopulations within femoral and tibial growth plates was examined by in situ hybridization at embryonic days E13.5-E18.5 and in early postnatal development (P0-P4). The levels and the patterns of type II collagen, *Ihh* and *Runx2* expression were unchanged in *Fosl2*$^{-/-}$ embryos (data not shown). At initiation of hypertrophy at E13.5, no significant difference in type X collagen expression was observed (Fig. 1F). However, zones of terminally differentiated, hypertrophic chondrocytes expressing type X collagen were consistently narrower in *Fosl2*$^{-/-}$ embryos during embryonic and early postnatal development (E14.5-P4; Fig. 1F).

Defective chondrocyte differentiation and extracellular matrix production

To further investigate the role of Fra2 on chondrocyte

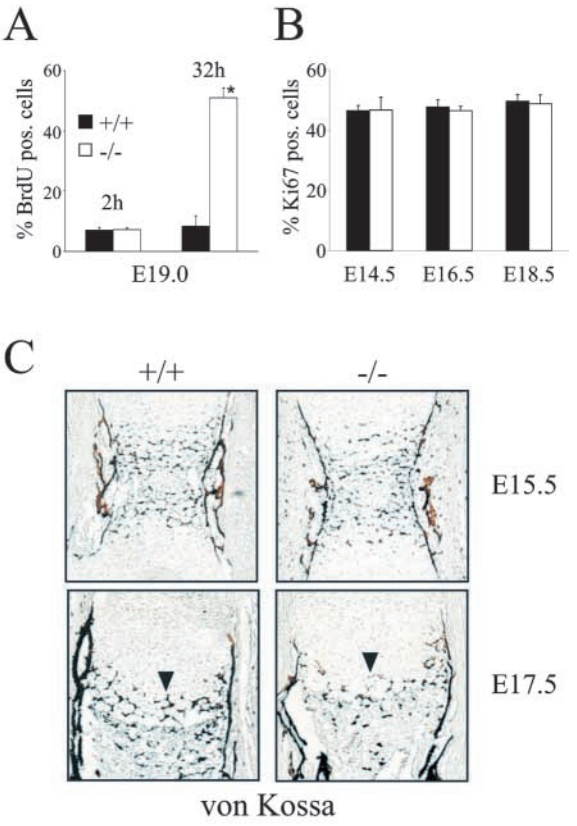

Fig. 2. Impaired chondrocyte differentiation and matrix deposition in vivo. (A) In vivo BrdU labeling of E19.0 embryos. The labeling index (BrdU positive cells/total cells) in zones of proliferating chondrocytes is shown. Bars represent mean value±s.d.; n=2; *P<0.001. (B) Ki67-positive cells in zones of proliferating chondrocytes at E14.5, E16.5 and E18.5. (C) Von Kossa staining of calcified matrix of E15.5 and E17.5 *Fosl2*$^{-/-}$ embryos and littermate controls. Arrowheads indicate stained matrix in the zones of hypertrophic chondrocytes. Pictures were taken at 50× magnification.

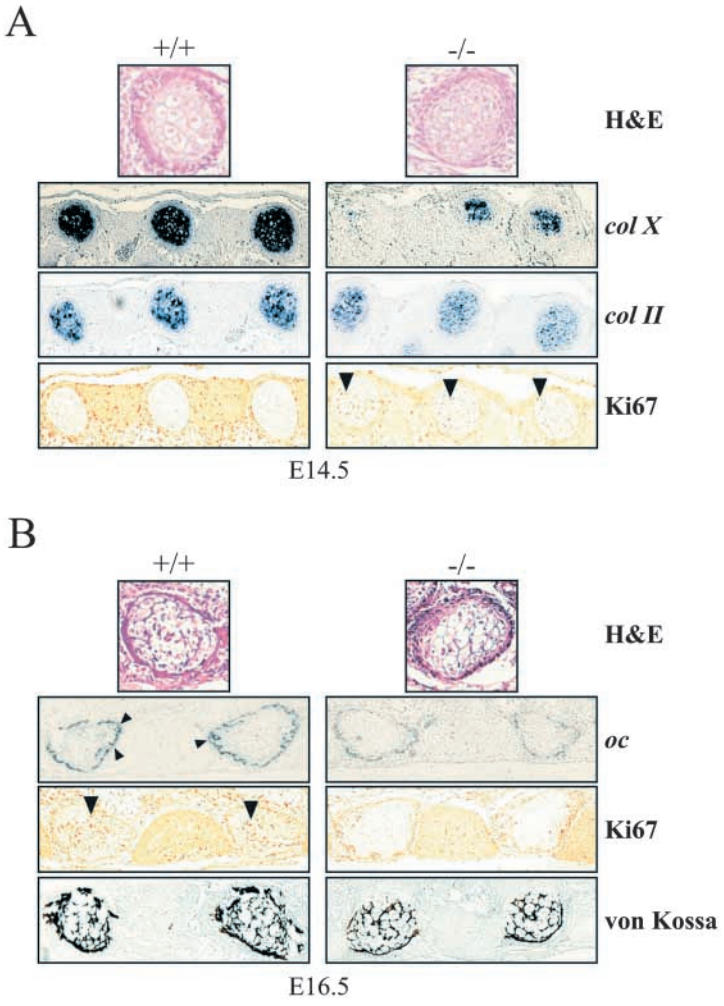

Fig. 3. Delayed development of vertebral columns in *Fosl2*$^{-/-}$ embryos. (A) Hematoxylin and Eosin staining, in situ hybridization using type II collagen (*col II*) and type X collagen (*col X*) antisense probes, and Ki67 immunohistochemistry on transversally sectioned primordial arches of developing vertebrae at E14.5. Arrowheads indicate Ki67-stained cells. (B) Hematoxylin and Eosin staining, in situ hybridization using *osteocalcin* (oc, arrowheads) antisense probe, Ki67 immunohistochemistry and von Kossa staining on transversally sectioned primordial arches of developing vertebrae at E16.5. Arrowheads indicate Ki67-stained cells. Pictures were taken at 100× (Haematoxylin and Eosin) and 50× (in situ hybridization, Ki67 immunohistochemistry, von Kossa staining) magnification.

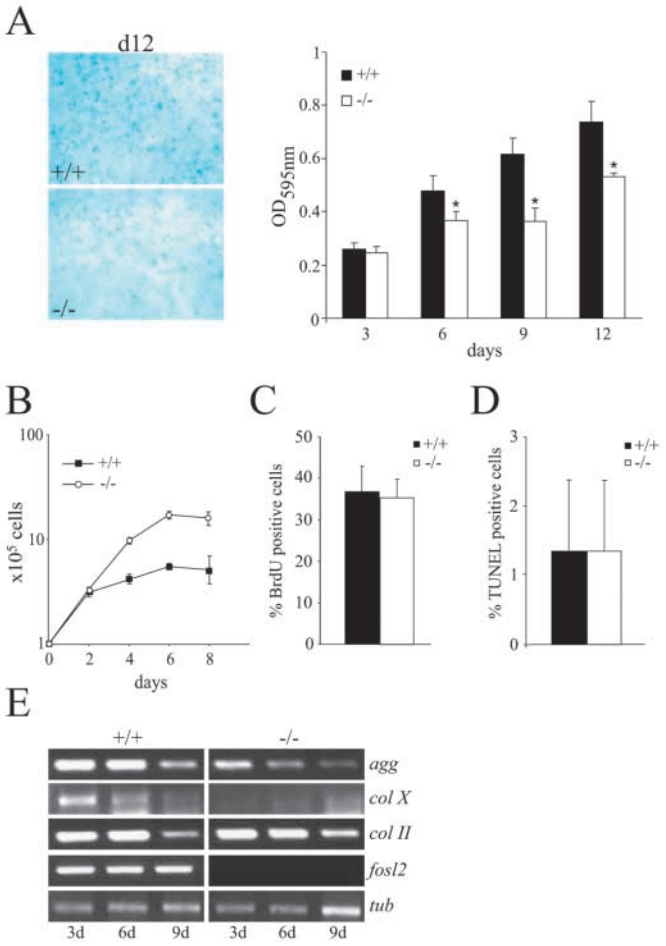

Fig. 4. Impaired differentiation and delayed senescence of primary *Fosl2*$^{-/-}$ rib cage chondrocytes. (A) Formation of cartilage nodules by primary chondrocytes. Primary chondrocyte cultures stained with Alcian Blue (left panel). Quantification of Alcian Blue bound to sulfated proteoglycans in the chondrocyte ECM (right panel). Bars represent mean value±s.d.; n=4; *P<0.05. (B) Cumulative cell number assay of primary rib cage chondrocytes. Experiments were carried out in triplicate and repeated three times. One representative experiment is shown. (C) BrdU-labeling of proliferating primary chondrocytes after 2 days in culture. (D) TUNEL staining of apoptotic primary chondrocytes after 2 days in culture. (E) Semi-quantitative RT-PCR analysis of cartilage markers and *Fosl2* in primary rib cage chondrocytes at day 3, 6 and 9 of differentiation. Tubulin was used as a loading control (n=3).

proliferation and differentiation, the fate of BrdU-labeled cells was examined in the growth plate. It has been shown that impaired chondrocyte differentiation leads, over time, to an accumulation of BrdU-labeled cells in the proliferating zone (Naski et al., 1998). Interestingly, the percentage of labeled cells in the zone of proliferating chondrocytes in *Fosl2*$^{-/-}$ growth plates 32 hours after BrdU injection was sixfold higher than in littermate controls, whereas no difference was observed 2 hours after BrdU injection (Fig. 2A). Numbers of Ki67-positive cells were unchanged during embryonic development (Fig. 2B), and no premature or increased apoptosis was observed in growth plates of *Fosl2*$^{-/-}$ embryos (data not shown). These findings suggest that impaired chondrocyte differentiation might lead to the reduced zones of hypertrophic chondrocytes in *Fosl2*$^{-/-}$ embryos and newborns. Impaired chondrocyte differentiation into mature hypertrophic chondrocytes may cause reduced ECM production in the epiphysis. At E15.5, we detected slightly reduced calcified matrix using von Kossa staining in *Fosl2*$^{-/-}$ long bones and the difference became more pronounced at E17.5, suggesting reduced extracellular matrix (ECM) deposition in long bones of *Fosl2*$^{-/-}$ embryos compared with control littermates (Fig. 2C).

Delayed hypertrophic differentiation in Fra2-deficient vertebrae

To examine whether Fra2 plays similar roles in the development of the axial and the appendicular skeleton, we investigated cartilage development in primordial arches of developing spines. At E14.5, cells of the primordial arches appeared smaller and less hypertrophic suggesting a delay in chondrogenesis (Fig. 3A). This observation was confirmed by markedly reduced type X collagen expression of *Fosl2*$^{-/-}$ chondrocytes in the primordial arches at E14.5, whereas no difference was observed in the expression pattern of type II collagen (Fig. 3A). The type II collagen probe used for this study shows a more intense staining in prehypertrophic chondrocytes, also arguing for reduced numbers of prehypertrophic chondrocytes owing to delayed differentiation in *Fosl2*$^{-/-}$ spines. In addition, *Fosl2*$^{-/-}$ chondrocytes of the developing vertebrae were positive for

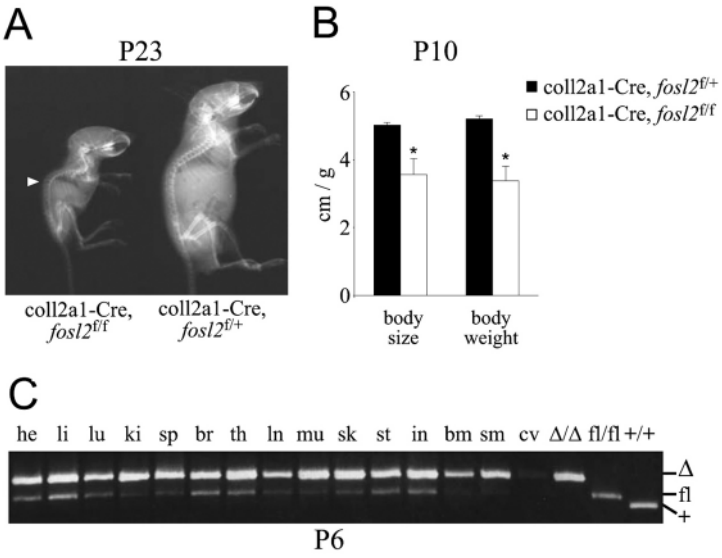

Fig. 5. Growth retardation in coll2a1-Cre, *Fosl2*$^{f/f}$ mice. (A) X-ray analysis of a 23-day-old coll2a1-Cre, *Fosl2*$^{f/f}$ mouse and littermate control (arrowhead indicates kyphosis). (B) Decreased body size and body weight of coll2a1-Cre, *Fosl2*$^{f/f}$ mice at P10. Bars represent mean value±s.d.; n=5; *P<0.05. (C) PCR for *Fosl2* deletion in tissues and organs of a 6-day-old coll2a1-Cre, *Fosl2*$^{f/f}$ mouse. The same deletion efficiency was observed in older mice. he, heart; li, liver; lu, lung; ki, kidney; sp, spleen; br, brain; th, thymus; ln, lymph node; mu, muscle; sk, skin; st, stomach; in, intestine; bm, bone marrow; sm, sternum; cv, calvariae.

Ki67 at E14.5. Thus, proliferating chondrocytes are still present, whereas wild-type cells had already ceased proliferation and started to differentiate into hypertrophic chondrocytes (Fig. 3A). At E16.5, expression of the osteoblast-specific marker osteocalcin was strongly decreased in primordial arches of *Fosl2*$^{-/-}$ embryos, whereas no difference in type X collagen expression was observed (Fig. 3B; data not shown). Moreover, Ki67-positive cells, which at this time point are likely to be proliferating osteoblasts, were present in wild-type, but absent in *Fosl2*$^{-/-}$ primordial arches (Fig. 3B). Furthermore, less calcified matrix had been deposited in vertebrae of *Fosl2*$^{-/-}$ embryos at E16.5 (Fig. 3B). At E18.5, no difference in both osteocalcin expression and numbers of Ki67-postive cells was observed (data not shown). These data indicate that initiation of endochondral ossification of the spine is delayed for 1-2 days.

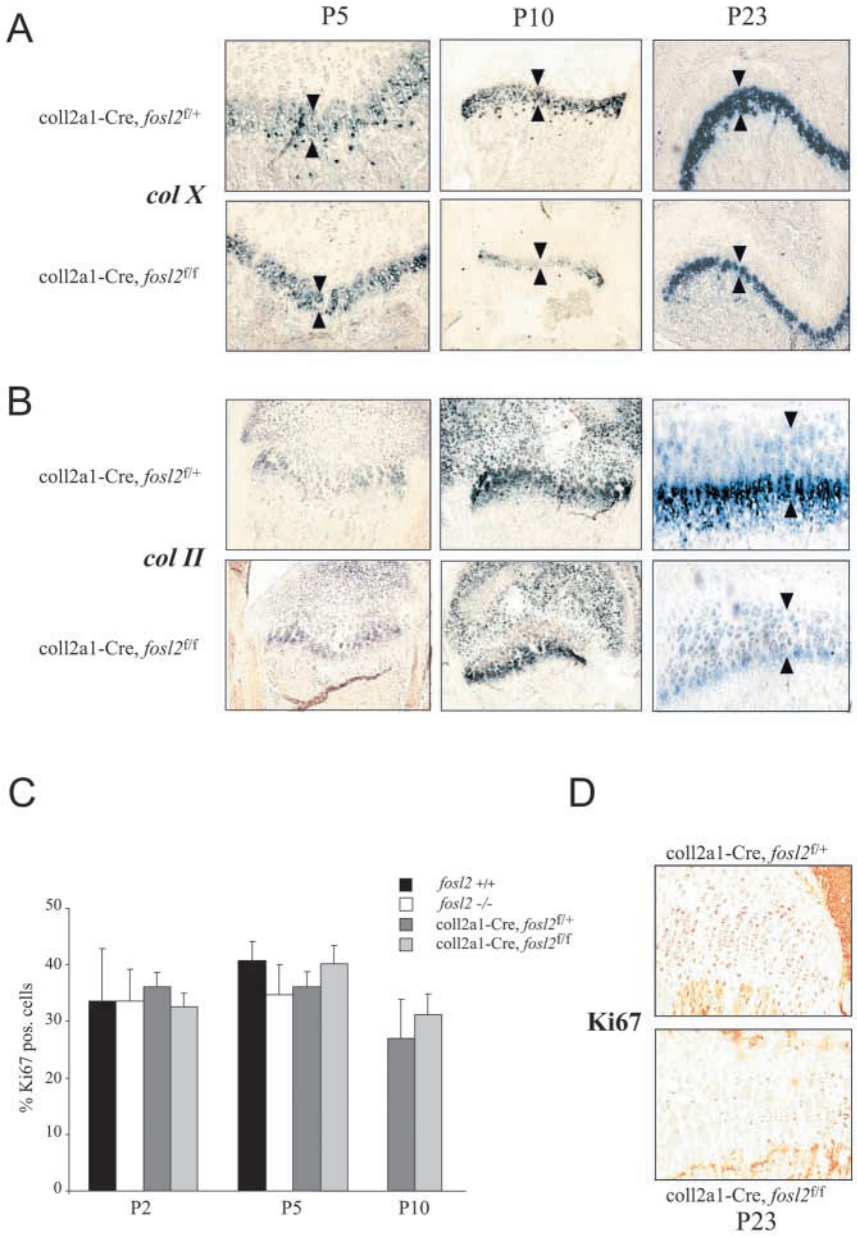

Fig. 6. Impaired hypertrophic differentiation but normal proliferation in coll2a1-Cre, *Fosl2*$^{f/f}$ mice. (A,B) In situ hybridization of femoral growth plates of P5, P10 and P23 coll2a1-Cre, *Fosl2*$^{f/f}$ mice and littermate controls using type X collagen (A) and type II collagen (B) antisense probes. Arrowheads indicate size of proliferating zone. (C) Ki67-positive cells in femoral growth plates of *Fosl2*$^{-/-}$, coll2a1-Cre, *Fosl2*$^{f/f}$ and control mice at P2, P5 and P10. Bars represent mean value±s.e.m.; n=3. (D) Immunohistochemical detection of Ki67 in P23 coll2a1-Cre, *Fosl2*$^{f/f}$ mice and P23 coll2a1-Cre, *Fosl2*$^{f/+}$ controls. Pictures were taken at 50× (*col X*, P10 and P23; *col II*, P5 and P10) and 100× (*col X*, P5; *col II*, P23; Ki67) magnification.

Impaired chondrocyte differentiation in vitro

To assess whether impaired differentiation of *Fosl2*$^{-/-}$ chondrocytes is due to a cell-autonomous defect, we isolated primary rib cage chondrocytes from newborn mice and assessed their proliferation and differentiation potential in culture. To investigate chondrocyte differentiation, *Fosl2*$^{-/-}$ and control cultures were stained with Alcian Blue. After 12 days in high-density differentiation cultures, the number of cells forming cartilage nodules and expressing ECM proteoglycans was reduced by approximately 30-50% in cultures of *Fosl2*$^{-/-}$ chondrocytes compared with wild-type cultures (Fig. 4A). The reduced differentiation of *Fosl2*$^{-/-}$ chondrocytes in vitro became evident after 6 days in culture (Fig. 4A). Interestingly, when cultured under subconfluent conditions for several passages, primary *Fosl2*$^{-/-}$ chondrocytes ceased proliferation and gained a large, flattened morphology later than wild-type chondrocytes (Fig. 4B; data not shown). However, numbers of proliferating and apoptotic chondrocytes were unchanged in wild-type and *Fosl2*$^{-/-}$ chondrocytes after 2 days in culture (Fig. 4C,D), resembling the in vivo situation. The expression of cartilage markers in vitro was assessed by semi-quantitative RT-PCR. The expression levels of the investigated cartilage marker genes declined during the culture period, which has been observed previously (Pavlov et al., 2003). Levels of hypertrophic chondrocyte ECM protein aggrecan was decreased and type X collagen expression was almost completely absent in differentiating *Fosl2*$^{-/-}$ chondrocytes, whereas expression of type II collagen was unchanged (Fig. 4E).

Chondrocyte differentiation and proliferation defects in conditional knockout mice lacking Fra2

As pups lacking Fra2 die during the first week after birth, we used a conditional approach to further characterize the cartilage defects during postnatal development. *Fosl2*$^{f/f}$ mice were crossed to coll2a1-Cre transgenic animals (Haigh et al., 2000). The coll2a1-Cre, *Fosl2*$^{f/f}$ mice were born at Mendelian ratio, but died between postnatal days (P) 10 and 25, and were severely growth retarded (Fig.

5A,B). At birth, however, coll2a1-Cre, *Fosl2*$^{f/f}$ pups were indistinguishable from their littermate controls and skeletal staining of newborn mice revealed normal skeleton architecture and bone size (data not shown). All coll2a1-Cre, *Fosl2*$^{f/f}$ mice developed a kyphosis-like abnormal bending of the vertebral column, starting between P5 and P10 (Fig. 5A, arrowhead). The coll2a1-Cre line was described to delete in cartilage, heart, developing eye, neuroepithelium, epidermis and cranial mesenchymal cells at E12.5 (Haigh et al., 2000). We found that *Fosl2* was partially deleted in most postnatal organs, implying that this Cre-line has a rather broad specificity in adult tissues (Fig. 5C).

We next characterized growth plate chondrocyte proliferation and differentiation in conditional *Fosl2* knockout mice. Consistent with the phenotype of *Fosl2*$^{-/-}$ embryos, coll2a1-Cre, *Fosl2*$^{f/f}$ mice showed a narrower zone of hypertrophic chondrocytes at all investigated postnatal time points (P5-P23) as assessed by in situ hybridization using a type X collagen antisense probe, and defective mineralization in zones of hypertrophic chondrocytes (Fig. 6A; data not shown). At postnatal day 5 and 10, in situ hybridization for type II collagen revealed normal zones of proliferating chondrocytes (Fig. 6B); however, at P23, coll2a1-Cre, *Fosl2*$^{f/f}$ mice showed a reduced zone of proliferating chondrocytes, most probably as a consequence of the general growth retardation. Ki67 staining revealed unchanged chondrocyte proliferation at P2, P5 and P10 (Fig. 6C). The percentage of proliferating chondrocytes in coll2a1-Cre, *Fosl2*$^{f/f}$ newborns was comparable with that of *Fosl2*$^{-/-}$ newborns, indicating that in both mouse models Fra2 deficiency has no impact on early postnatal chondrocyte proliferation. However, at P23, the number of Ki67-positive cells in conditional knockout mice was reduced (Fig. 6B), probably secondary to the general severe growth retardation because of the absence of Fra2 in other organs.

Discussion

In this study, we show that Fra2 is a novel transcription factor required for cartilage development. Although skeletal staining revealed no obvious malformations in *Fosl2*$^{-/-}$ embryos and newborns, we observed slightly smaller mineralized regions in long bones and narrower zones of hypertrophic chondrocytes. This might be due to impaired differentiation into hypertrophic chondrocytes, where Fra2 is normally expressed (Carrasco and Bravo, 1995). Moreover, endochondral ossification was delayed in spines of *Fosl2*$^{-/-}$ embryos. After birth, *Fosl2*$^{-/-}$ pups are growth retarded and survive up to 5 days postnatally. The lethality is probably due to defects in hearts and/or gastrointestinal tracts of *Fosl2*$^{-/-}$ newborns (R.E., unpublished).

Fra2 deficiency led to narrower hypertrophic zones in femoral and tibial growth plates and this correlated with reduced expression of the chondrocyte markers type X collagen and aggrecan in vivo. Impaired proliferation or increased apoptosis of growth plate chondrocytes did not contribute to the shortened hypertrophic zones in *Fosl2*$^{-/-}$ embryos. As BrdU-labeled cells accumulated in proliferation zones of *Fosl2*$^{-/-}$ embryos in a time-dependent manner, impaired differentiation into hypertrophic chondrocytes most probably caused the shortened hypertrophic zones. Besides Fra2 expression in chondrocytes, Fra2 has been shown to be expressed in dividing and apoptotic rat calvarial osteoblasts, but is even higher expressed in differentiating osteoblasts (McCabe et al., 1995) and becomes the principal Fos protein in fully differentiated osteoblasts in vitro (McCabe et al., 1996). This suggests a role for Fra2 in differentiation of mesenchyme-derived cells. We could not observe any differences in expression of typical cartilage markers, such as type II collagen, Runx2 and Ihh. Moreover, resting and proliferating zones had unchanged sizes, indicating that Fra2 very specifically affects chondrocyte differentiation. As *Fosl2*$^{-/-}$ chondrocytes fail to differentiate properly, their matrix deposition was reduced. This might impair the bone-forming activity, as less scaffold is provided, leading to decreased bone mass. The function of Fra2 in the interplay between cartilage and osteoblasts and osteoclasts remains to be determined. Interestingly, the related Fos family member Fra1 has recently been shown to also regulate bone mass through bone matrix production by osteoblasts and chondrocytes (Eferl et al., 2004).

Primary cultures of *Fosl2*$^{-/-}$ rib cage chondrocytes displayed impaired differentiation in vitro, consistent with the in vivo finding. In addition, *Fosl2*$^{-/-}$ chondrocytes continued to proliferate and gained a large, flattened morphology later when compared with wild-type cells. The in vitro differentiation defect was accompanied by markedly reduced expression of the hypertrophic chondrocyte-specific marker type X collagen and to a lesser extent aggrecan. However, Fra2 does not bind to the AP1 site in the type X collagen promoter (Harada et al., 1997) in EMSAs (data not shown), suggesting that Fra2 might not directly regulate ECM protein expression. Direct regulation of ECM proteins might be accomplished by Fos, as it has been described to mediate PTH/PTHrP regulated type X collagen expression in chicken (Ionescu et al., 2001; Riemer et al., 2002). Expression of other markers that affect chondrogenesis, such as FGFR3, FGF18, FGF9, bone morphogenetic protein 4 (BMP4), PTHrP, PTH/PTHrP receptor (PPR), Ihh, Runx2, alkaline phosphatase and TGFβ was found unchanged in vitro (data not shown). Fra2 seems to be an inducer of hypertrophic differentiation and its absence might keep chondrocytes in a proliferative state, which might contribute to reduced chondrocyte differentiation in vivo and in vitro. Presumably, Fra2 can mediate signals elicited by Ihh/PTHrP or FGF, which control cartilage differentiation (Kronenberg and Chung, 2001; Coumoul and Deng, 2003), although the pathway and the target genes of Fra2 remain to be determined.

We also employed a conditional approach to investigate postnatal cartilage development. Unfortunately, we found Fra2 deleted to some extent in most organs of coll2a1-Cre, *Fosl2*$^{f/f}$ mice, indicating that this Cre mouse strain is not chondrocyte-specific in postnatal tissues. Nevertheless, we found deletion of Fra2 in the cartilage of the sternum and we were able to describe a cartilage phenotype. Similar to *Fosl2*$^{-/-}$ embryos, coll2a1-Cre, *Fosl2*$^{f/f}$ mice showed a reduced zone of hypertrophic chondrocytes, but at later postnatal stages suffered from an abnormal dorsoventral bending of the vertebral column (kyphosis). Interestingly, both *Fosl2*$^{-/-}$ and coll2a1-Cre, *Fosl2*$^{f/f}$ mice are growth retarded after P1 but show no growth defect during embryogenesis. The reduced hypertrophic differentiation in Fra2-deficient cartilage is likely to account for the observed growth retardation, as it has been

described in other studies with defective hypertrophic chondrocyte differentiation (Horiki et al., 2004).

Chondrocyte proliferation appears to be insensitive to the effects of Fra2 deficiency during embryonic and early postnatal development, as we observed no differences in *Fosl2*$^{-/-}$, coll2a1-Cre, *Fosl2*$^{f/f}$ and wild-type mice until P10. However, only 1-5% of coll2a1-Cre, *Fosl2*$^{f/f}$ mice survive until weaning, and we found reduced chondrocyte proliferation and a reduction in the size of the zone of proliferating chondrocytes in femoral growth plates of these mice at P23. Fra2 might therefore have no effect on chondrocyte proliferation during early (pre-weaning), but a role in proliferation at later (post-weaning) stages of bone development. This has also been reported for other factors involved in bone development, e.g. FGFR3 (Naski et al., 1998) and insulin-like growth factor 1 (IGF1) (Baker et al., 1993; Liu et al., 1993). However, the broad spectrum of Fra2 deletion in coll2a1-Cre, *Fosl2*$^{f/f}$ mice suggests that the proliferation defect in post-weaning mice might simply be secondary to the severe growth retardation and to profound defects in various Fra2 deficient organs, which most probably also accounts for the lethality.

In developing vertebral bodies of *Fosl2*$^{-/-}$ embryos, endochondral ossification appears to be delayed for 1-2 days. At E14.5, differentiation into hypertrophic chondrocytes, and at E16.5, osteoblast invasion and matrix production are markedly reduced. Moreover, Fra2 deficiency in the spine of coll2a1-Cre, *Fosl2*$^{f/f}$ mice leads to kyphosis, a skeletal malformation. An abnormal bending of the spine was also observed when *Jun* was conditionally deleted using coll2a1-Cre transgenic mice (Behrens et al., 2003), suggesting that Fra2 and Jun might form the predominant AP1 dimer in vertebral development. In contrast to *Fosl2*$^{-/-}$ spines, endochondral ossification is not delayed in long bones and no malformations were observed in the appendicular skeletons of *Fosl2*$^{-/-}$ mice, indicating different functions of Fra2 in the development of the appendicular and axial skeleton.

In conclusion, mice lacking the transcription factor Fra2 display reduced chondrocyte differentiation throughout development, which leads to growth retardation postnatally, and delayed endochondral ossification of the spine. The identification of Fra2 target genes during chondrocyte differentiation may lead to a better understanding of pathways that coordinate cartilage development.

We are grateful to C. Hartmann for providing in situ hybridization probes and helpful discussions. We also thank A. Grigoriadis, R. Gruber, C. Hartmann, D. Mehic and J. Witowsky for critical reading of the manuscript. A.H. is a recipient of an EMBO and Marie Curie Individual fellowship. The IMP is funded by Boehringer Ingelheim and this work was partly supported by the Austrian Industrial Research Promotion Fund.

References

Baker, J., Liu, J. P., Robertson, E. J. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75**, 73-82.

Behrens, A., Haigh, J., Mechta-Grigoriou, F., Nagy, A., Yaniv, M. and Wagner, E. F. (2003). Impaired intervertebral disc formation in the absence of Jun. *Development* **130**, 103-109.

Carrasco, D. and Bravo, R. (1995). Tissue-specific expression of the fos-related transcription factor fra-2 during mouse development. *Oncogene* **16**, 1069-1079.

Cohen, M. M., Jr (2002). Some chondrodysplasias with short limbs: molecular perspectives. *Am. J. Med. Genet.* **112**, 304-313.

Coumoul, X. and Deng, C. X. (2003). Roles of FGF receptors in mammalian development and congenital diseases. *Birth Defects Res. Part C Embryo Today* **69**, 286-304.

Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* **84**, 911-921.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-754.

Eferl, R., Hoebertz, A., Schilling, A. F., Rath, M., Karreth, F., Kenner, L., Amling, M. and Wagner, E. F. (2004). The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J.* **23**, 2789-2799.

Fleischmann, A., Hafezi, F., Elliott, C., Reme, C. E., Ruther, U. and Wagner, E. F. (2000). Fra-1 replaces c-Fos-dependent functions in mice. *Genes Dev.* **14**, 2695-2700.

Foletta, V. C., Sonobe, M. H., Suzuki, T., Endo, T., Iba, H. and Cohen, D. R. (1994). Cloning and characterisation of the mouse fra-2 gene. *Oncogene* **9**, 3305-3311.

Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A. and Wagner, E. F. (1994). c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* **266**, 443-448.

Haigh, J. J., Gerber, H. P., Ferrara, N. and Wagner, E. F. (2000). Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. *Development* **127**, 1445-1453.

Harada, S., Sampath, T. K., Aubin, J. E. and Rodan, G. A. (1997). Osteogenic protein-1 up-regulation of the collagen X promoter activity is mediated by a MEF-2-like sequence and requires an adjacent AP-1 sequence. *Mol. Endocrinol.* **11**, 1832-1845.

Hess, J., Hartenstein, B., Teurich, S., Schmidt, D., Schorpp-Kistner, M. and Angel, P. (2003). Defective endochondral ossification in mice with strongly compromised expression of JunB. *J. Cell Sci.* **116**, 4587-4596.

Hilberg, F., Aguzzi, A., Howells, N. and Wagner, E. F. (1993). c-jun is essential for normal mouse development and hepatogenesis. *Nature* **365**, 179-181.

Horiki, M., Imamura, T., Okamoto, M., Hayashi, M., Murai, J., Myoui, A., Ochi, T., Miyazono, K., Yoshikawa, H. and Tsumaki, N. (2004). Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy and causes dwarfism with osteopenia. *J. Cell Biol.* **165**, 433-445.

Ionescu, A. M., Schwarz, E. M., Vinson, C., Puzas, J. E., Rosier, R., Reynolds, P. R. and O'Keefe, R. J. (2001). PTHrP modulates chondrocyte differentiation through AP-1 and CREB signaling. *J. Biol. Chem.* **276**, 11639-11647.

Jochum, W., David, J. P., Elliott, C., Wutz, A., Plenk, H., Jr, Matsuo, K. and Wagner, E. F. (2000). Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. *Nat. Med.* **6**, 980-984.

Jochum, W., Passegue, E. and Wagner, E. F. (2001). AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401-2412.

Johnson, R. S., van Lingen, B., Papaioannou, V. E. and Spiegelman, B. M. (1993). A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* **7**, 1309-1317.

Karsenty, G. (1999). The genetic transformation of bone biology. *Genes Dev.* **13**, 3037-3051.

Karsenty, G. and Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2**, 389-406.

Kenner, L., Hoebertz, A., Beil, T., Keon, N., Karreth, F., Eferl, R., Scheuch, H., Szremska, A., Amling, M., Schorpp-Kistner, M. et al. (2004). Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J. Cell Biol.* **164**, 613-623.

Kobayashi, T., Chung, U. I., Schipani, E., Starbuck, M., Karsenty, G., Katagiri, T., Goad, D. L., Lanske, B. and Kronenberg, H. M. (2002). PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. *Development* **129**, 2977-2986.

Komori, T. (2002). Runx2, a multifunctional transcription factor in skeletal development. *J. Cell Biochem.* **87**, 1-8.

Kronenberg, H. M. and Chung, U. (2001). The parathyroid hormone-related protein and Indian hedgehog feedback loop in the growth plate. *Novartis Found. Symp.* **232**, 144-152.

Li, Y. and Olsen, B. R. (1997). Murine models of human genetic skeletal disorders. *Matrix Biol.* **16**, 49-52.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. and Efstratiadis, A.

(1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75**, 59-72.

Liu, Z., Xu, J., Colvin, J. S. and Ornitz, D. M. (2002). Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev.* **16**, 859-869.

McCabe, L. R., Kockx, M., Lian, J., Stein, J. and Stein, G. (1995). Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. *Exp. Cell Res.* **218**, 255-262.

McCabe, L. R., Banerjee, C., Kundu, R., Harrison, R. J., Dobner, P. R., Stein, J. L., Lian, J. B. and Stein, G. S. (1996). Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. *Endocrinology* **137**, 4398-4408.

Murtaugh, L. C., Chyung, J. H. and Lassar, A. B. (1999). Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* **13**, 225-237.

Naski, M. C., Colvin, J. S., Coffin, J. D. and Ornitz, D. M. (1998). Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development* **125**, 4977-4988.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N. and Takada, S. (2002). FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev.* **16**, 870-879.

Pavlov, M. I., Sautier, J. M., Oboeuf, M., Asselin, A. and Berdal, A. (2003). Chondrogenic differentiation during midfacial development in the mouse: in vivo and in vitro studies. *Biol. Cell.* **95**, 75-86.

Riemer, S., Gebhard, S., Beier, F., Poschl, E. and von der Mark, K. (2002). Role of c-fos in the regulation of type X collagen gene expression by PTH and PTHrP: localization of a PTH/PTHrP-responsive region in the human COL10A1 enhancer. *J. Cell Biochem.* **86**, 688-699.

Ruther, U., Komitowski, D., Schubert, F. R. and Wagner, E. F. (1989). c-fos expression induces bone tumors in transgenic mice. *Oncogene* **4**, 861-865.

Sabatakos, G., Sims, N. A., Chen, J., Aoki, K., Kelz, M. B., Amling, M., Bouali, Y., Mukhopadhyay, K., Ford, K., Nestler, E. J. et al. (2000). Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat. Med.* **6**, 985-990.

Schorpp-Kistner, M., Wang, Z. Q., Angel, P. and Wagner, E. F. (1999). JunB is essential for mammalian placentation. *EMBO J.* **18**, 934-948.

St-Jacques, B., Hammerschmidt, M. and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13**, 2072-2086.

Thomas, D. P., Sunters, A., Gentry, A. and Grigoriadis, A. E. (2000). Inhibition of chondrocyte differentiation in vitro by constitutive and inducible overexpression of the c-fos proto-oncogene. *J. Cell Sci.* **113**, 439-450.

Wang, Z. Q., Grigoriadis, A. E., Mohle-Steinlein, U. and Wagner, E. F. (1991). A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. *EMBO J.* **10**, 2437-2450.

Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U. and Wagner, E. F. (1992). Bone and haematopoietic defects in mice lacking c-fos. *Nature* **360**, 741-745.