Defective endochondral ossification in mice with strongly compromised expression of JunB

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Summary

Functional analysis in mice has established an absolute requirement of JunB, a member of the AP-1 transcription factor family, during early embryonic development. To investigate the role of JunB during mid and late gestation and postnatally Ubi-JunB transgenic mice were used to generate two junB−/− Ubi-JunB mutant lines, in which embryonic lethality was rescued but strongly reduced JunB expression in several adult tissues was observed. Mutant mice from both rescue lines were growth retarded and shared significantly reduced longitudinal bone growth. Mutant long bones were characterised by reduced numbers of growth plate chondrocytes and a severe osteoporosis. Decreased JunB levels in epiphysal growth plate chondrocytes and bone lining osteoblasts correlated with deregulated expression of Cyclin A, Cyclin D1 and p16INK4a, key regulators of cell cycle control. Furthermore, junB−/− Ubi-JunB bone marrow stromal cells were unable to differentiate into bone forming osteoblasts in vitro. Our data demonstrate that JunB plays a crucial role in endochondral ossification by regulating proliferation and function of chondrocytes and osteoblasts.

Supplemental data available online

Key words: AP-1, Bone, Chondrocytes, Cell cycle, Osteoblasts, Osteoporosis

Introduction

The skeletal system is a dynamic connective tissue consisting of cartilage and bone. The two major processes that control skeletogenesis are endochondral and intramembranous ossification. In membranous skeletal tissue, condensed mesenchymal cells directly differentiate into osteoblasts, whereas in endochondral skeletal elements, mesenchymal cells differentiate into chondrocytes to form a cartilage scaffold that will subsequently be replaced by bone. Thus formation of the skeleton by endochondral ossification requires a carefully orchestrated interplay of at least three major cell types: chondrocytes, osteoblasts and osteoclasts (Karsenty and Wagner, 2002).

Growth of endochondral bones is controlled by a variety of extracellular factors such as hormones, growth factors and cytokines, which co-ordinate the proliferation and differentiation of growth plate chondrocytes (Mundlos and Olsen, 1997a; Mundlos and Olsen, 1997b). However, the transcription factors that mediate altered gene expression during these processes are still poorly understood (Karsenty, 1999). Knowledge about transcriptional regulators that are critically involved in bone formation mainly originates from mouse models where transcription factor genes are ablated or overexpressed (Karsenty and Wagner, 2002; McCauley, 2001). One transcription factor with essential function in skeletogenesis is the activator protein-1 (AP-1) (Jochum et al., 2001). AP-1 is a dimeric transcription factor formed by Jun (c-Jun, JunB, and JunD), Fox (c-Fos, Fra-1, Fra-2, and FosB), and ATF proteins (ATF-2 and ATF-3) and is involved in diverse biological processes such as differentiation, proliferation, cell survival and transformation (Angel and Karin, 1991; Karin et al., 1997). The various subunits of AP-1 can be induced at sites of active bone formation in vivo by transforming growth factor-β (TGF-β), parathyroid hormone (PTH), and 1,25-dihydroxy vitamin D regulating osteoblast proliferation and differentiation (Karsenty, 1999). In this context, a functional interaction between AP-1 and Runx2 has been demonstrated during PTH-dependent MMP13 expression in osteoblasts (D’Alonzo et al., 2002; Hess et al., 2001). Runx2 is a member of the Runt family of transcription factors and is one of the most important players during osteoblast differentiation (Ducy et al., 2000; Karsenty and Wagner, 2002). Besides its essential function in osteoblastogenesis, in vivo models have demonstrated that it is also required for hypertrophic chondrocyte differentiation (Takeda et al., 2001).

Recently, genetic studies in mice have provided insight into the role of several AP-1 family members in skeletal development in vivo. Transgenic mice overexpressing c-Fos develop chondro- and osteosarcomatous lesions (Grigoriadis et al., 1993; Wang et al., 1991), whereas mice lacking c-Fos develop osteopetrosis because of an early block of differentiation in the osteoblast lineage (Grigoriadis et al., 1994; Jacenko, 1995). Impaired differentiation of osteoclasts in the absence of c-Fos is rescued by Fra-1, a Fos-related protein encoded by the c-Fos target gene fosl (Matsuo et al., 2000). Furthermore, transgenic mice overexpressing Fra-1 or
ΔFosB, a splice variant of FosB, exhibit increased bone formation, apparently by different but yet unknown mechanisms (Jochum et al., 2000; Sabatakos et al., 2000). Another AP-1 family member that contributes to endochondral ossification, is ATF-2. In ATF-2-deficient mice chondrocyte proliferation is reduced, causing dwarfism and skeletal deformities (Reimold et al., 1996). Recently cyclin D1 and cyclin A, which are involved in cell cycle progression, have been identified as ATF-2 target genes in chondrocytes (Beier et al., 1999; Beier et al., 2000). Additionally, overexpression of Jun family members has also been shown to perturb the maturation of chondrocytes (Kameda et al., 1997). All these data together show that several AP-1 family members are crucial for proper differentiation and function of both cartilage- and bone-forming cells of the skeleton.

Mice deficient for AP-1 family members c-Jun, JunB and Fra-1 are embryonic lethal, or display decreased postnatal viability, i.e., ATF-2 (Jochum et al., 2001; Schorpp-Kistner et al., 2001). Therefore, functional analysis of these AP-1 family members in the process of bone formation and bone remodelling requires establishment of more complex and conditional mouse models. To investigate the role of JunB during mid and late gestation and postnatal processes, Ubi-junB transgenic mice that express JunB under the control of the human ubiquitin C promoter were used to generate junB–/– Ubi-junB mutant lines, in which embryonic lethality was rescued (Hartenstein et al., 2002; Passegue et al., 2001; Schorpp-Kistner et al., 1999). Recently, we have established two junB–/– Ubi-junB lines, which exhibit strongly reduced transgenic expression in several tissues of adult mice (Hartenstein et al., 2002). Both lines showed growth retardation, severe osteoporosis and a hypochondrodysplasia-like phenotype. These phenotypes correlated with reduced proliferation of bone marrow stromal cells in vitro as well as growth plate chondrocytes and osteoblasts in vivo. Impaired proliferation might be explained by deregulated expression of cell cycle regulators Cyclin A, Cyclin D1 and p16INK4a that have been demonstrated to be potential JunB target genes (Passegue and Wagner, 2000; Bakiri et al., 2000; Andrecht et al., 2002).

Materials and Methods

Generation of mutant animals

Generation and genotyping of junB–/– mice as well as Ubi-junB transgenic mice (288, 311) have been described previously (Hartenstein et al., 2002; Schorpp-Kistner et al., 1999). All animals were housed in specific pathogen free (SPF) environment and under light, temperature and humidity controlled conditions. Food and water were available ad libidum. The procedures for performing animal experiments were in accordance with the principles and guidelines of the ATBW (officials for animal welfare) and were approved by the Regierungspräsidium Karlsruhe, Germany.

RNA preparation and RT-PCR analysis

Total RNA from the epiphysis of long bones or cultured bone marrow cells were prepared using the peqGold RNAPure™ kit (PeqLab, Erlangen, Germany) according to the manufacturer’s protocol. To reduce bones to small pieces we used the Ultra Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). For RT-PCR 2 µg total RNA pre-treated with RQ-DNAse (Promega, Mannheim, Germany) were reverse transcribed using AMV reverse transcriptase (Promega) in 20 µl reactions, as recommended by the manufacturer.

PCR was performed using 2-3 µl cDNA and the following primers: junB-B48 5'-CGAAGCGCGCTGCGAGAA-3’ and junB-B18 5'-TCAAAGTGGGGGCTCCGATG-3’, cyclin A for 5’-CCCGAGAATGCAGATTTGTG-3’ and cyclin A rev 5’-CATGGTTGGCGCCTTTGAGTAGG-3’, cyclin D1 for 5’-TGCCGCTGCCCAAAAGTTA-3’ and cyclin D1 rev 5’-CTGTAAGAGATAAAGAAGATC-3’, p16 for 5’-CGGGATCCGCTGCA-GACAGCTGGCCAG-3’ and p16 rev 5’-GAACCTTAGGCA GCCGCACATCCACG-3’, or β-tubulin-specific primers described elsewhere (Rohwedel et al., 1995).

Histological analysis

For histological analysis tissues were fixed in PBS-buffered 4% paraformaldehyde, embedded in paraffin, sectioned at 6 µm and stained using standard techniques. Calciﬁed bones were decalcified in 0.5 M EDTA pH 7.4 for 10-14 days before embedding. TRAP staining was done as described previously (Gack et al., 1995).

In situ hybridisation

For in situ hybridisation, 6 µm paraffin sections from decalcified bones were treated and hybridised with 35S-UTP-labelled sense and antisense probes as described previously (Tuckermann et al., 2000). JunB cRNA sense and anti-sense probes were derived by in vitro transcription from an EcoRI or BamHI linearised plasmid containing a 159 bp fragment (nucleotides 1266-1425 of the published sequence). Cyclin A cRNA sense and antisense probes were derived by in vitro transcription from an Apal and SacI linearised plasmid containing a 400 bp fragment (nucleotides 915-1315 of the published sequence). Mouse osteopontin was ampliﬁed by PCR using primers described previously (Guo et al., 2001) and cloned in the pGem(T) vector (Promega). The following probes have been described previously: MMP-13, MMP-9, ALP, VEGF, osteocalcin, type-I a1, type-II and type-X collagen (Gack et al., 1995; Tuckermann et al., 2000; Schorpp-Kistner et al., 1999).

Immunohistochemistry

Immunohistochemistry was performed on 6 µm sections with monoclonal anti-PCNA antibody (1:25; DAKO, Hamburg, Germany), monoclonal anti-Jun-B-c11 antibody (1:25, sc-8051, Santa Cruz Biotechnology, California, USA) using the MOM kit (Vector Laboratories, Burlingame, USA) and polyclonal anti-Cyclin A antibody (1:100, sc-751, Santa Cruz Biotechnology) and polyclonal anti-p16 antibody (1:200, sc-1207, Santa Cruz Biotechnology) using the Vectastain kit (Vector laboratories) according to the manufacturer’s instructions. Sections were counterstained with Haematoxylin.

In vitro proliferation and differentiation of marrow stromal cells

Bone marrow cells were extruded from tibiae and femurs of 4- to 6-week-old mice, and cultured in α-MEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 15% foetal calf serum (Sigma, Taufkirchen, Germany) at 37°C and 6% CO2 which was changed every 2-3 days. For in vitro differentiation, nearly confluent cells were trypsinized and replated in 48-well plates at a density of 2-3×104 cells/well. Next day the medium was replaced with α-MEM medium supplemented with 10% foetal calf serum, 50 µg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma). ALP activity and mineral deposition were analysed as described previously (Enomoto et al., 2000; Schiller et al., 2001). In vitro proliferation, bone marrow cells were cultured for 10 days in α-MEM supplemented with 15% foetal calf serum. Cells were trypsinized and replated in 96-well plates at a density of 1×104 cells/well or in 60 mm dishes to allow growth on coverslips. The number of cells in the 96-well plates was measured at indicated time points using a Coulter Z2 counter.
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(Beckman Coulter, Krefeld, Germany). Labelling with BrdU and immunohistological analysis have been described elsewhere (Andrecht et al., 2002). Colony forming unit assay was carried out as described elsewhere (Li et al., 2000).

**Results**

junB−/− Ubi-junB mice show growth retardation and shorter long bones

Recently, we generated two junB−/− Ubi-junB lines – 288R and 311R – in which embryonic lethality of JunB-deficient embryos was rescued (Schorpp-Kistner et al., 1999; Passegue et al., 2001), but there was strongly reduced transgenic activity in adult tissues (Hartenstein et al., 2002). Owing to significant transgene expression throughout embryogenesis, mutant embryos did not exhibit obvious abnormalities (data not shown). Four weeks after birth junB−/− Ubi-junB mice were phenotypically distinct with visibly shorter bodies compared to sex- and age-matched wild-type and junB+/+ Ubi-junB (288K or 311K) control transgenic littermates. The overall decrease in body size became even more significant in 8-week-old junB−/− Ubi-junB mice (Fig. 1A) and correlated with reduced length of individual appendicular long bones (Fig. 1B and data not shown). As 311R mice showed even lower transgenic expression in all tested adult tissues compared to 288R mice (Hartenstein et al., 2002), most of the data presented here were derived from the 311R line.

Growth retardation of mutant mice combined with decreased length of long bones suggested that reduced JunB expression affected endochondral bone growth. To measure JunB levels in bone of control and 311R mice, we performed RT-PCR analysis of total RNA prepared from epiphysis of 8-week-old mice (Fig. 1C). The amount of junB mRNA in bones from mutant mice was strongly reduced compared to controls. The same result was also obtained by an RNAse protection assay demonstrating that the amount of transgenic mRNA in bone of 288R and 311R mice was around 10% and 1%, respectively, of the endogenous level expressed in wild-type, 288K and 311K transgenic control mice (data not shown). As expected from previous studies in lymphoid cells (Hartenstein et al.,

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**Fig. 1.** Compromised JunB expression in mutant mice leads to growth retardation and shortening of long bones. (A) Comparison of an 8-week-old control (311K; bottom) and a junB−/− Ubi-junB (311R; top) mouse, (B) reduced length of tibiae and femurs of age matched female junB−/− Ubi-junB (311R; open circles) compared to control mice (filled circles). Black bars indicate the mean values of control (n=10, wt and 311K) and mutant animals (n=7), (C) RT-PCR analysis of junB mRNA with total RNA derived from long bone epiphysis of 8-week-old junB−/− Ubi-junB mice (311R) and control (311K) littermates. RT-PCR analysis for β-Tubulin served as a control for quality and quantity of the cDNA samples. (D) Expression of JunB protein was detected by immunohistochemistry in proliferative (PC) and prehypertrophic chondrocytes, but not in hypertrophic chondrocytes (HC), on 6 μm growth plate sections from 4-week-old control (311K) mice. Additionally, expression of JunB protein was demonstrated in control osteoblasts and osteocytes in the trabecular bone area (arrows). In junB−/− Ubi-junB bone sections only very few chondrocytes, but no osteoblasts, were positive for JunB protein (arrows). Control sections were incubated only with the second antibody (left panel). Sections were counterstained with Haematoxylin; magnification was 20×.
no significant differences in the amount of functional \textit{junB} transcripts were observed in bone of wild-type, 288K and 311K control mice (data not shown). On the cellular level \textit{junB} transcripts and protein were most prominent in growth plate chondrocytes of 4-week-old control mice, but we could also detect \textit{junB} mRNA and protein in bone-lining osteoblasts and osteocytes of femur and tibia bone sections (Fig. 1D and data not shown). Compared to control sections JunB levels were greatly reduced in mutant bones and only a very small percentage of cells in the growth plate stained positive for JunB protein. Furthermore, expression in osteoblasts and osteocytes was undetectable in long bone sections of \textit{junB}−/− Ubi-\textit{junB} mice (Fig. 1D).

Reduced size of growth plates and decreased number of proliferative chondrocytes in \textit{junB}−/− Ubi-\textit{junB} long bones

In order to analyse the growth defect in greater detail, we examined tibiae and femoral bone sections of control and mutant animals. The overall size of the growth plate was reduced in 4- and 8-week-old \textit{junB}−/− Ubi-\textit{junB} mice compared to control littermates and the reduction was still detectable in bones of 16-week-old animals (Fig. 2A). Detection of \textit{type-II} and \textit{type-X collagen} mRNA by in situ hybridisation revealed a dramatic reduction in proliferative and prehypertrophic chondrocytes in mutant growth plates, whereas the number of hypertrophic chondrocytes was only marginally decreased compared to controls (Fig. 2B). From these data, we concluded that compromised JunB expression in \textit{junB}−/− Ubi-\textit{junB} mice affects proliferation rather than differentiation of growth plate chondrocytes. Comparable number of hypertrophic chondrocytes expressing \textit{alkaline phosphatase} (\textit{alp}), \textit{vascular endothelial growth factor} (\textit{vegf}) or \textit{osteopontin} (\textit{opn}) mRNA in control and mutant growth plates further supported this conclusion (Fig. 2B; see also Fig. 4G-I). Additionally, we did not detect any increase in TUNEL-positive growth plate chondrocytes in 4- and 8-week-old bone sections of \textit{junB}−/− Ubi-\textit{junB} mice excluding the possibility that enhanced apoptosis is responsible for the observed phenotype (data not shown).

\textit{junB}−/− Ubi-\textit{junB} mice develop osteoporosis

In addition to the effects in growth plate chondrocytes, \textit{junB}−/− Ubi-\textit{junB} long bones were characterised by severe osteoporosis. Loss of trabecular bone was detectable in long bones of 4-week-old mutant animals and became even more prominent with age (Fig. 3A). Furthermore, we found

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**Fig. 2.** Hypochondrodysplasia-like phenotype in growth plates of \textit{junB}−/− Ubi-\textit{junB} mice. (A) Growth plate morphology of proximal tibiae from 4-, 8- and 16-week-old control (311K) and \textit{junB}−/− Ubi-\textit{junB} (311R) littermates. Black lines indicate differences in the size of control and mutant growth plates. Sections were stained with Haematoxylin and Eosin. PC, proliferative chondrocytes; HC, hypertrophic chondrocytes. Magnification was 20× (4 weeks, 8 weeks) and 40× (16 weeks). (B) In situ detection of \textit{type-II} (\textit{col-II}), \textit{type-X collagen} (\textit{col-X}), alkaline phosphatase (\textit{alp}) and \textit{vascular endothelial growth factor} (\textit{vegf}) mRNAs in growth plates of femur sections prepared from 4-week-old control (middle panel) and \textit{junB}−/− Ubi-\textit{junB} (right panel) littermates. Hybridisation of control sections with sense probes served as control for specificity (left panel). Dark-field images were used to demonstrate \textit{vegf} expression in hypertrophic chondrocytes. Sections were counterstained with Haematoxylin and Eosin; magnification was 20×.
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significant reduction of cortical bone on tibiae and femoral sections from 16-week-old junB+/− Ubi-junB animals compared to controls (Fig. 3A). Reduced bone thickness was also observed in mutant bones of the skull (data not shown) suggesting that altered expression of JunB affects both endochondral and intramembranous ossification.

Since we did not detect an increase in the number of osteoclasts in mutant mice, as measured by expression of the osteoclast-specific marker tartrate resistant acid phosphatase (TRAP) and mmp-9 mRNA (Fig. 3B,C), impaired trabecular bone formation is best explained by defective osteoblast development and/or function. To gain experimental support for this, in vivo osteoblastogenesis was determined by in situ detection of osteoblast-specific marker genes on femoral and tibiae sections of 4-week-old mice. Loss of trabecular bone in junB−/− Ubi-junB mice correlated with a slight decrease in the number of cells expressing type-I collagen mRNA (early differentiation marker), but overall we could detect osteoblasts in mutant bones expressing several markers specific for terminally differentiated osteoblasts such as MMP-13, Osteopontin and Osteocalcin (Fig. 4). Remarkably, in mutant mice osteoblasts expressing osteocalcin mRNA were enriched at the chondrocyte-osteoblast junction (Fig. 4).

In vitro differentiation of bone marrow-derived stromal cells into ALP-positive osteoblasts was slightly reduced in mutant cultures compared to controls (Fig. 5A). Moreover, Alizarin Red staining of in vitro differentiated stromal cells from control and junB−/− Ubi-junB bone marrow revealed considerably reduced bone nodule formation in junB−/− Ubi-junB cultures (Fig. 5A) suggesting that JunB is required for osteoblast function in vitro.

Since we observed a slightly reduced number of type-I collagen-positive osteoblasts in mutant long bones, we addressed the question of whether a decrease in the number of osteoprogenitor cells contributes to the observed phenotype in bone formation. We performed colony-forming unit assays (CFU) measuring ALP-positive colonies, which arise 3 weeks after plating bone marrow cells at low density. Bone marrow cultures from junB−/− Ubi-junB mice showed a significant decrease in ALP-positive and ALP-negative colonies compared to control cultures (Fig. 5B). Moreover, a reduction in colony size was observed because of lower cell number per colony (Fig. 5B), suggesting a general growth defect in mutant marrow stromal cells. In line with this assumption a reduced growth rate of bone marrow-derived mutant stromal cells was observed (Fig. 6A). The reduced growth rate of mutant cells was most probably due to defective proliferation, as demonstrated by BrdU incorporation and immunohistochemical analysis of PCNA expression (Fig. 6B and data not shown).

Deregulated expression of cell cycle proteins in chondrocytes and osteoblasts

JunB-deficient mouse embryo fibroblasts are characterised by defective cell cycle control due to deregulated expression of cell cycle regulators, such as Cyclin A, Cyclin D1 and p16INK4a (Passegue and Wagner, 2000; Bakiri et al., 2000; Andrecht et al., 2002). Recently, it has been demonstrated that Cyclin A and
Cyclin D1 play an important role in chondrocyte proliferation and that promoter activation depends on the AP-1 family member ATF-2 (LuValle and Beier, 2000). In agreement with the deregulated expression in JunB-deficient mouse fibroblasts, cultured $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bone marrow cells showed increased expression of $\text{cyclin D1}$ mRNA and decreased expression of $\text{cyclin A}$ mRNA compared to control cells (Fig. 6C). Furthermore, mutant cells showed significantly higher levels of $\text{p16}$ mRNA, which could counteract the proliferative activity of increased Cyclin D1 expression (Fig. 6C). Deregulated expression of these cell cycle regulators correlated with loss of JunB expression in $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bone marrow cells as compared to high $\text{junB}$ mRNA levels observed in control cells (Fig. 6C).

Impaired proliferation of chondrocytes and osteoblasts as a result of altered expression of cell cycle regulators could explain the observed defect in endochondral ossification of $\text{junB}^{-/-}$ Ubi-$\text{junB}$ long bones. This assumption was supported by the expression pattern of the proliferation marker PCNA in 4-week-old control and mutant bone sections. In control sections almost all chondrocytes in the type-II collagen area and some of the bone lining osteoblasts were PCNA positive (Fig. 7B,D). In $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bone sections the number of PCNA-positive chondrocytes and osteoblasts was significantly reduced, while the number of PCNA-positive haematopoietic cells in the bone marrow cavity was similar to controls (Fig. 7C,E). Corresponding to our in vitro data, a reduced number of proliferative cells was associated with decreased expression of $\text{cyclin A}$ mRNA and protein as well as increases in p16 protein levels in chondrocytes and osteoblasts of $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bone sections compared to controls (Fig. 7F-O and data not shown).

**Discussion**

Gain-of-function and loss-of-function investigations of the gene of interest in an animal model is at present the most effective tool contributing to recent advances in skeletal biology (Karsenty and Wagner, 2002; McCauley, 2001; McLean and Olsen, 2001). In this study, we used transgenic mice, which express JunB under the control of the human ubiquitin C promoter to rescue embryonic lethality of $\text{junB}$ deficiency (Schorpp-Kistner et al., 1999). Two mutant mouse lines were generated with significant transgene expression during embryogenesis, which became greatly reduced in tissues of adult mutant mice, such as the haematopoietic system, bones and skin (Hartenstein et al., 2002) (unpublished data). These mutant animals served as a tool to investigate the role of JunB during bone development and remodeling in adults, and also its potential role in disease.
JunB is required for proliferation rather than differentiation of growth plate chondrocytes

Both mutant mouse lines were growth retarded and characterised by reduced length of long bones. Longitudinal bone growth depends on the co-ordinated interplay of chondrocyte proliferation, hypertrophy and apoptosis (White and Wallis, 2001). Within the growth plate the various subpopulations of chondrocytes are arranged in characteristic columns resulting from the rapid proliferation of chondrocytes within the constraints of the cartilaginous matrix. In junB mutant animals, the size of the endochondral growth plate was significantly reduced. The reduction was obvious for all subpopulations of chondrocytes but most prominent for the zone of type-II collagen-positive proliferative cells, suggesting that decreased numbers of hypertrophic chondrocytes might be a consequence of defective proliferation. However, we cannot exclude the possibility that JunB is also involved in the regulation of chondrocyte differentiation.

During recent years, various growth factors and hormones have been implicated in the regulation of chondrocytic proliferation and differentiation, including FGFs, PTHrP, Indian hedgehog (Ihh) and C-type natriuretic peptide (CNP) (Karsenty and Wagner, 2002). JunB might influence chondrogenesis either by transcriptional control of proteins involved in signalling or by regulation of JunB expression...
and/or function in response to these signalling pathways. Although, in areas of proliferative and hypertrophic chondrocytes we found altered expression levels of factors involved in PTH/PTHrP and Ihh signalling, such as Ihh and PTH/PTHrP receptor (data not shown), this is most probably because of changes in the number of chondrocytes rather than direct effects on transcriptional regulation by JunB. However, it is well established that AP-1 activity and expression of different AP-1 members changes in response to extracellular signals. Interestingly, in the nervous system CNP was found to rapidly induce \textit{junB} mRNA expression in vivo and in vitro (Thiriet et al., 1997; Thiriet et al., 2001). CNP belongs to the natriuretic peptide family and is expressed in many cell types including chondrocytes. In vitro, CNP favours chondrocyte proliferation and longitudinal growth of foetal bones, whereas CNP-deficient mice develop severe dwarfism as a result of impaired endochondral ossification (Chusho et al., 2001). It will be interesting to investigate whether CNP signalling also influences JunB expression in chondrocytes thereby regulating proliferation and/or differentiation.

Yet, what are the potential JunB target genes regulating chondrocyte proliferation? Consistent with recent findings that \textit{cyclin A} and \textit{cyclin D1} are direct JunB targets (Andrecht et al., 2002; Bakiri et al., 2000), we observed reduced levels of \textit{cyclin A} mRNA and increased \textit{cyclin D1} mRNA expression in mutant bone marrow culture serving as a source for progenitors of chondrocytic and osteoblastic cells. Recently, altered \textit{cyclin D1} and \textit{cyclin A} mRNA levels and function have been shown to be responsible for reduced chondrocyte proliferation causing dwarfism and skeletal deformities in ATF-2-deficient mice (Beier et al., 1999; Beier et al., 2000). In line with the published role of JunB and ATF2 in \textit{cyclin A} and \textit{cyclin D1} regulation, our in vivo and in vitro studies provide supporting evidence that JunB, possibly in collaboration with ATF2, regulates expression of these genes in chondrocytes and osteoblasts, respectively.

In addition to deregulated expression of Cyclin A and Cyclin D1, we observed an up-regulation of the CDK inhibitor p16\textsuperscript{INK4a} in \textit{junB}–/– Ubi-\textit{junB} bone marrow stromal cell cultures, which might explain impaired proliferation in the presence of higher Cyclin D1 levels. Previous findings in fibroblasts have demonstrated that JunB suppresses cell proliferation in fibroblasts and induces premature senescence by transcriptional activation of p16\textsuperscript{INK4a} (Passegue and Wagner, 2000). Yet, our data strongly suggest that transcriptional regulation of p16\textsuperscript{INK4a} mediated by JunB seems to depend on the cell type and context.
Deregulated expression of Cyclin A and p16INK4a proteins was confirmed in mutant growth plate chondrocytes and osteoblasts suggesting that impaired cell cycle regulation is in part responsible for the bone phenotype observed in $\text{junB}^{-/-}$ Ubi-$\text{junB}$ mice.

Role of JunB in osteoblastogenesis and bone formation

Beside the abnormal chondrogenesis, mutant animals displayed an osteoporosis phenotype with reduced trabecular and cortical bone. Additionally, bones formed by intramembranous ossification such as calvaria were also reduced in thickness (data not shown). Although we have not performed in-depth histomorphometric analysis to quantify the osteoporotic phenotype in mutant bones, the present data (i) qualitative changes in bone architecture, (ii) our preliminary findings in teratomas derived from wild-type and JunB-deficient ES cells where no sign of osteoblast development or mineralised areas in the absence of JunB could be observed (unpublished data) and (iii) impaired in vitro differentiation of bone marrow-derived mutant stromal cell cultures strongly support that JunB function contributes to osteoblastogenesis. This is in line with recent studies showing that AP-1 components have important functions in the regulation of osteoblast proliferation and differentiation (Iochum et al., 2001; St-Arnaud and Quelo, 1998). In osteoblasts, AP-1 activity can be induced by BMPs, TGF-β, PTH and 1,25-dihydroxy vitamin D, all of which are potent regulators of osteogenesis (Karsenty, 1999). BMP-2 and TGF-β have been shown to specifically up-regulate the expression of JunB and c-fos mRNAs in osteoblasts, and this increase was correlated in both cases with an enhanced AP-1 DNA-binding activity involving JunB and c-Fos proteins (Paley et al., 2000). Furthermore, PTH treatment of mice resulted in increased mRNA expression of JunB and other AP-1 members in vivo (Stansilas et al., 2000).

In addition to cell-autonomous defects in JunB-compromised osteoblasts, impaired angiogenesis as a direct or indirect consequence of reduced JunB expression may contribute to reduced trabecular bone in mutant animals (Gerber and Ferrara, 2000; Bianco and Robey, 2000). In fact, preliminary data revealed a reduced number of blood vessels in $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bone sections (data not shown). Corresponding to reports that angiogenesis is functionally linked to apoptosis induction in hypertrophic chondrocyte (Gerber and Ferrara, 2000), reduced blood vessel formation in 16-week-old mutant long bones correlates with an accumulation of type-X collagen-positive chondrocytes (unpublished data). Angiogenesis is well known to be a prerequisite for endochondral ossification and the vascularisation is considered as a source of bone marrow stromal cells that are progenitors of skeletal tissue components (Gerber and Ferrara, 2000). A direct link between JunB expression and angiogenesis has been demonstrated in JunB-deficient embryos, in which defective neovasculature correlates with reduced expression of VEGF, VEGF-R1 and MMP-9 (Schorpp-Kistner et al., 1999).

Analysing bone sections of control and mutant mice by in situ hybridisation, we did not observe major difference in Vegf expression. In light of recent findings providing direct evidence that angiogenesis mediated by VEGF signalling and MMP-9 activity is essential for the regulation of trabecular bone formation (Gerber and Ferrara, 2000), it will be interesting to analyse whether the observed reduction in MMP-9 expressing cells contributes to the phenotype in $\text{junB}^{-/-}$ Ubi-$\text{junB}$ bones.

In summary, we demonstrate that JunB plays a crucial role in endochondral ossification by regulating proliferation and function of chondrocytes and osteoblasts. Since the observed reduction in trabecular bone could be partially explained by the hypochondrodysplasia phenotype, in the future additional experimental models, preferably conditional knockout mice, are needed to clarify the function of JunB in the osteoblast lineage.

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