Impaired ossification in mice lacking the transcription factor Sp3

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Abstract

Sp3 is a ubiquitously expressed member of the Sp family of transcription factors. Recently, the mouse *Sp3* gene has been disrupted by homologous recombination. Sp3 *null* mice die immediately after birth due to respiratory failure. In addition, these mice show a pronounced defect in late tooth formation. Here we show that Sp3 is also required for proper skeletal ossification. Both endochondral and intramembranous ossification are impaired in E18.5 Sp3−/− embryos. The delay in ossification is reflected by reduced expression of the osteoblast-specific marker gene osteocalcin. The transcription factor – core binding factor 1 (Cbfa1) – that is essential for bone formation, however, is expressed at normal levels. In vitro differentiation studies using Sp3−/− ES cells further support the conclusion that Sp3 is needed for correct bone formation. The capacity of Sp3−/− cells to undergo osteogenic differentiation in vitro is reduced and *osteocalcin* expression is significantly diminished. Our studies establish Sp3 as an essential transcription factor for late bone development. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Sp3; Transcription factor; Knockout mice; Bone formation; Osteocalcin; Ossification; Skeleton; Osteoblast; ES cells; Embryoid bodies

1. Introduction

Many promoters and enhancers of housekeeping, tissue-specific and viral genes contain functionally important GC-rich DNA motifs. These motifs are recognized by a large family of transcription factors designated as the Sp/XKLF family of transcription (Philipsen and Suske, 1999). The Sp/XKLF family of proteins includes the Sp transcription factors (Sp1, Sp2, Sp3, Sp4, Sp5) characterized by a highly conserved DNA-binding domain at the C-terminus (Harrison et al., 2000; Suske, 1999; Treichel et al., 2001). Sp1, Sp3 and Sp4 are most closely related to each other, manifested by their ability to bind the classical GC-box (GGGCGGG) as well as related motifs with identical affinity. In addition, these three proteins contain glutamine-rich activation domains adjacent to serine/threonine-rich stretches in their N-terminal region. Sp2 also contains glutamine-rich activation domains, but fails to recognize the classical GC-box (Kingsley and Winoto, 1992). Vice versa, Sp5 lacks the glutamine-rich activation domain, but is able to bind the GC-box in vitro (Harrison et al., 2000).

Targeted mutations of the mouse genes encoding Sp1, Sp3, Sp4 and Sp5 have been reported (Bouwman et al., 2000; Harrison et al., 2000; Marin et al., 1997; Supp et al., 1996). Sp5 *null* mice show no overt phenotype (Harrison et al., 2000), whereas two-third of the Sp4 knockouts die within the first few days after birth. Viable Sp4−/− animals are smaller and the males do not reproduce (Supp et al., 1996). The strongest phenotypes are observed in Sp1 and Sp3 mutant mice. *Sp1* targeted embryos die at embryonic day E10 (Marin et al., 1997). Sp3 *null* embryos are retarded in growth and die invariably at birth of respiratory failure. The molecular cause of death is unclear since only minor morphological alterations were observed in the lung, and surfactant protein expression is indistinguishable from that in wild type mice. In addition, Sp3−/− mice show a pronounced defect in late tooth development. The dentin/enamel layer of the developing teeth is impaired due to the lack of the ameloblast-specific gene products amelogenin and ameloblastin (Bouwman et al., 2000).

Here we show that bone formation is impaired in Sp3−/− mice. Several ossification centres are completely absent in E18.5 Sp3 *null* embryos. The delay in ossification is reflected by reduced expression of the osteoblast-specific marker gene *osteocalcin*. In vitro differentiation studies show further that the capacity of ES cells to differentiate to osteoblasts in vitro is strongly reduced in the absence of

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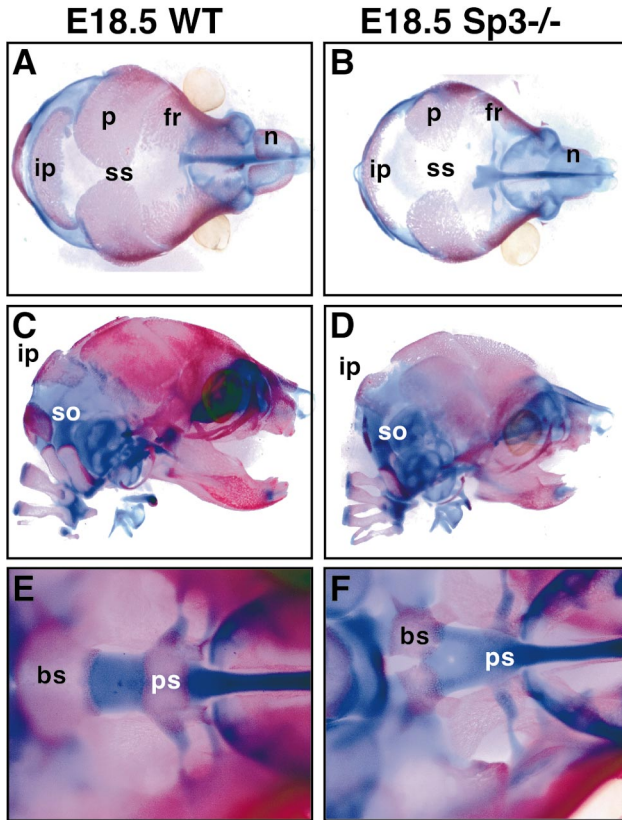*E-mail address:* suske@imt.uni-marburg.de (G. Suske).

Fig. 1. Skull preparations of wild type (A,C,E) and Sp3−/− (B,D,F) E18.5 embryos stained with Alcian Blue (cartilage) and Alizarin Red (bone). (A,B) Top view of the skull showing incomplete ossification of the intraparietal (ip), parietal (p), frontal (fr) and nasal (n) bone in Sp3-deficient mice (B) in comparison to their wild type (A) littermates. The saggital suture (ss) is wide open in Sp3−/− mice. (C,D) Lateral view of the skull exhibiting differences in the development of the supraoccipetal bone (so). (E,F) Ventral view of the caudal part of the skull showing that the basisphenoid bone (bs) is not closed in Sp3−/− embryos (F). Complete absence of an ossification centre is evident in the presphenoid bone (ps) of Sp3 knockout mice (F).

Sp3. Our studies establish a role for Sp3 in late bone development.

2. Results

2.1. Impaired ossification in Sp3−/− mice

Visual inspection of Sp3-deficient E18.5 embryos obtained by Caesarean section occasionally showed embryos with slight deformations of the skull. Most significantly, several embryos contained a swelling at the roof of the skull. In addition, perceptual inspection revealed a tender consistence of the whole embryo. These observations prompted us to examine cartilage and bone development in Sp3−/− mice.

Skeletons of wild type and Sp3−/− E18.5 embryos were prepared, and cartilage and bone visualized by Alcian Blue and Alizarin Red staining, respectively. These analyses

revealed several ossification defects in all parts of the Sp3−/− animals. The skull of E18.5 Sp3-deficient embryos showed an abnormally wide saggital suture (Fig. 1A,B). Ossification of the frontal, parietal, interparietal, nasal and supraoccipital bones was significantly reduced (Fig. 1A–D). In addition, ossification of several bones of the skull base was also affected. The presphenoid bone of Sp3 *null* mice exhibited an almost complete absence of mineralized bone tissue and the basisphenoid bones were not closed (Fig. 1E,F).

In the trunk region, hypo-ossification of the sternebrae, the vertebral bodies and the atlas dens was evident in Sp3-deficient mice (Fig. 2A–F). The strongest difference was observed in the xiphoid process (Fig. 2G,H). Here, Sp3−/− mice lack completely any ossification centre.

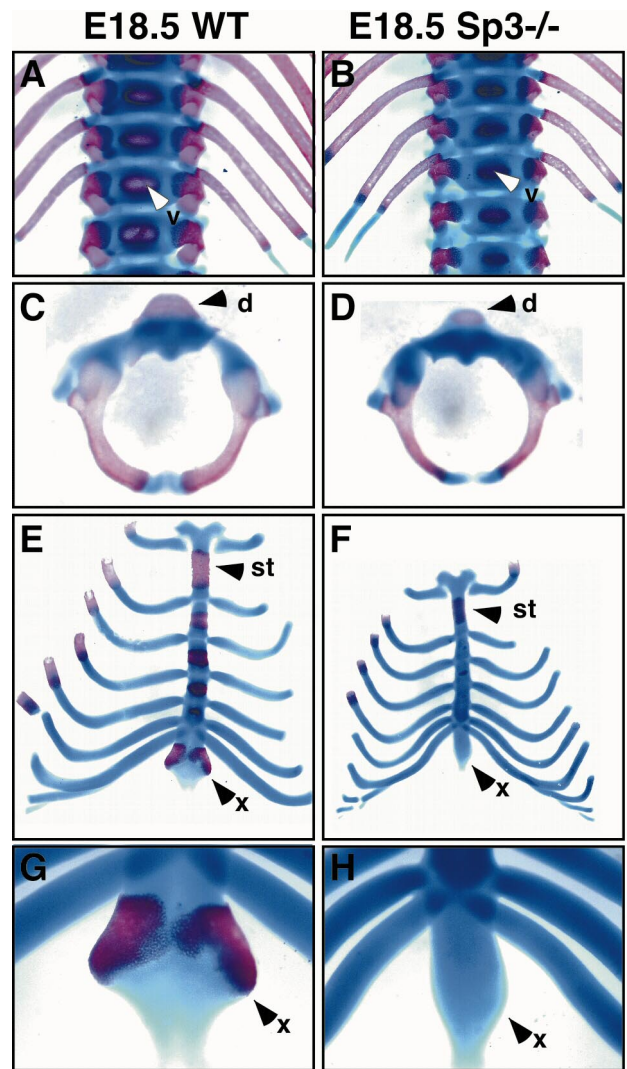
Fig. 2. Trunk preparations of wild type (A,C,E,G) and Sp3−/− (B,D,F,H) E18.5 embryos stained with Alcian Blue (cartilage) and Alizarin Red (bone). Ventral view of the backbone (A,B) and the rib-cage (E,F). The atlas (first cervical vertebra) is shown in (C) and (D). A higher magnification of the processus xiphoideus is shown in (G) and (H). Abbreviations are: d, dens; st, sternebrae; v, vertebral body; x, xiphoid process.

Inspection of the hind and fore paws (Fig. 3) exhibited shorter primary ossification centres in the metacarpals and metatarsi. In the distal and proximal phalanges bone formation was completely absent.

Taken together, these findings show that Sp3−/− embryos have several ossification abnormalities during late bone development. Cartilage formation, however, appears to be unaffected by the absence of Sp3.

2.2. Expression of the osteocalcin gene is impaired in Sp3−/− embryos

Osteoblasts are the bone forming cells that express genes that are required to form the mineralized tissue. We asked whether the altered skeletal ossification pattern of Sp3-deficient embryos could be linked to reduced expression of the osteoblast-specific gene products osteopontin and/or osteocalcin. Osteopontin is an acidic glycoprotein secreted at the onset of mineralization (Mark et al., 1988). Besides osteoblasts, several epithelial tissues, such as kidney tubules and uterine epithelium, synthesize osteopontin (Nomura et al., 1988). Osteocalcin, however, is highly bone-specific. It is expressed exclusively in mature osteoblasts during mineralization (Heinrichs et al., 1995). Osteocalcin is found in the mineralized matrix and facilitates the differentiation of osteoclasts (Glowacki et al., 1991; Liggett et al., 1994).

Northern blot analyses were performed with RNA extracted from newborn mice. Transcripts encoding osteopontin were detectable at similar levels in wild type and Sp3−/− mice (Fig. 4A). Thus, the expression of this gene is not down-regulated in Sp3−/− animals. In contrast, osteocalcin mRNA was readily detectable at birth in wild type mice but was almost absent in Sp3-deficient mice (Fig.
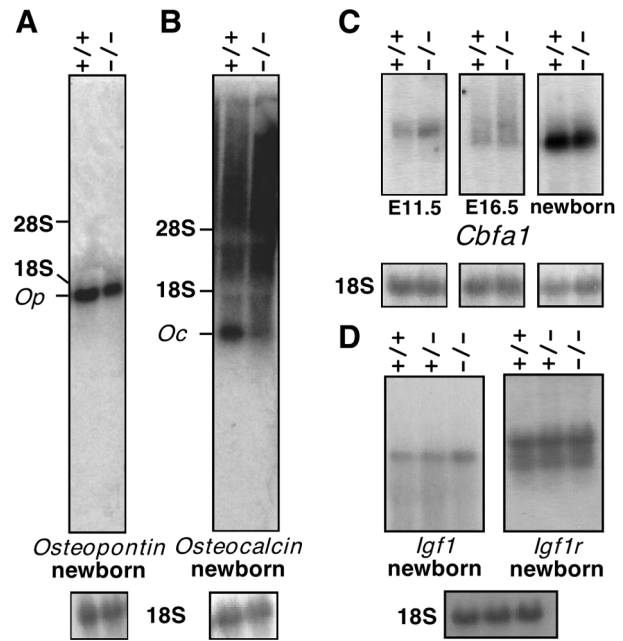

Fig. 4. Expression of putative Sp3 target genes during bone formation. RNA was extracted from the head of wild type (+/+), Sp3 +/− (+/−), and Sp3-deficient (−/−) embryos or newborn animals as indicated, subjected to electrophoresis through 1.2% formaldehyde-agarose gels and transferred to nylon membranes. The filters were hybridized with cDNA fragments encoding (A) osteopontin (Op), (B) osteocalcin (Oc), (C) core binding factor 1 (Cbfa1) and (D) insulin-like growth factor 1 (Igf1) and insulin-like growth factor 1 receptor (Igf1r). As a control the filters were probed with an 18S rRNA-specific oligonucleotide (18S).

4B). Thus, delayed ossification in Sp3−/− mice is reflected by strongly reduced expression of the osteocalcin gene.

2.3. Expression of Cbfa1, Igf1 and Igf1r are not affected in Sp3−/− mice

Our skeleton analysis suggested a delay of bone formation in Sp3-deficient embryos. Thus, we examined next the presence of transcripts encoding proteins regulating bone formation.

A central regulator of osteoblast differentiation and bone development is the transcription factor Cbfa1 (reviewed in Ducy, 2000). Disruption of the Cbfa1 gene resulted in complete lack of bone formation (Komori et al., 1997; Otto et al., 1997). Northern blot analyses of RNA from E11.5, E16.5 and newborn mice were performed. These experiments revealed that expression of the Cbfa1 gene was not affected in Sp3-deficient mice (Fig. 4C).

A developmental delay of the appearance of ossification centres was described in Igf1r-deficient mice (Liu et al., 1993). Therefore, we analyzed the expression of the Igf1r gene and its ligand Igf1. Transcripts encoding Igf1 and Igf1r were detectable at similar intensities in wild type, heterozygous and Sp3−/− newborn mice (Fig. 4D).

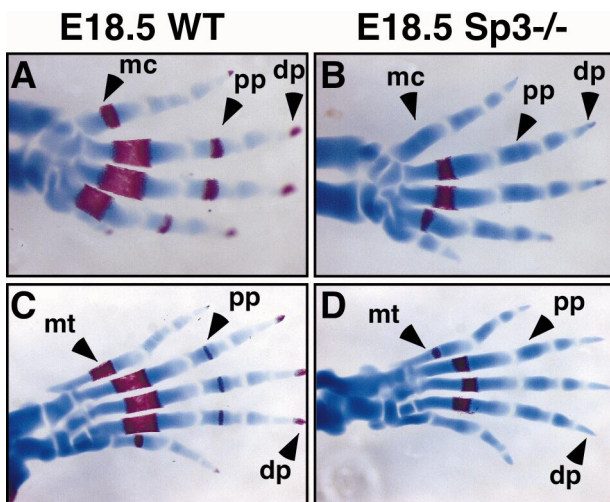Taken together, delayed ossification in Sp3−/− mice cannot be directly linked to reduced expression of the tran-

Fig. 3. Limb preparations of wild type (A,C) and Sp3−/− (B,D) of E18.5 embryos stained with Alcian Blue (cartilage) and Alizarin Red (bone). Both distal and proximal phalanges (dp and pp, respectively) of the fore paw (A,B) and hind paw (C,D) of normal embryos contain ossification centres completely lacking in their Sp3−/− counterparts (indicated by arrowheads). Metacarpals (mc) and metatarsal (mt) bones show reduced ossification.

scription factor *Cbfa1* or the insulin-like growth factor receptor gene *Igf1r* and its ligand *Igf1*.

2.4. Reduced capacity of Sp3−/− ES cells to undergo osteoblast differentiation

Our analyses of Sp3-deficient embryos showed that Sp3 contributes to late bone formation. Newborn Sp3−/− embryos exhibit only incomplete formation of mineralized bone tissue and expression of the osteoblast-specific protein osteocalcin is strongly reduced. To establish further a role of Sp3 in bone formation, we analyzed the capacity of recently established Sp3−/− embryonic stem (ES) cells (to be published elsewhere) to undergo osteogenic differentiation in vitro. ES cells are pluripotent cells retaining the capacity to generate any cell type in vivo and in vitro (Keller, 1995; Martin, 1981). Culture conditions to differentiate ES cells into osteoblasts at a high rate have been established recently (Phillips et al., 2001).

Wild type Sp3 + /− and Sp3−/− ES cells were allowed to form embryoid bodies (EB), then treated with retinoic acid, replated and maintained in osteogenic differentiation medium. The occurrence of mineralization nodules was determined by von Kossa staining. As shown in Fig. 5A, Sp3−/− EBs contain significantly fewer mineralization nodules than wild type derived EBs. Quantitative evaluation revealed that only 15% of differentiated Sp3−/− EBs contained mineralization nodules, in contrast to 60% of wild type and heterozygous Sp3 + /− EBs (Fig. 5B). We also tested the capacity of wild type and Sp3−/− EBs to undergo cardiomyocyte differentiation and obtained similar numbers of beating cardiomyocytes independent of the presence or absence of Sp3 (Fig. 5C). Thus, the reduced capacity of Sp3−/− EBs to undergo osteogenesis is not due to a general default of differentiation of Sp3−/− cells but is rather specific for certain cell lineages.

We also examined expression of the osteoblast-specific proteins osteocalcin and osteopontin by RT-PCR analyses of RNA obtained from differentiated ES cells. These experiments revealed that expression of *osteopontin* was not affected in Sp3−/− mutant cells (data not shown). Expression of *osteocalcin*, however, was strongly reduced in differentiated Sp3-deficient EBs (Fig. 5D). Altogether, our results show that the capacity to undergo osteogenesis is significantly reduced in Sp3-deficient EBs as compared to wild type or Sp3 + /− EBs.

3. Discussion

3.1. Bone formation is impaired in Sp3-deficient mice

Previous studies have shown that Sp3 is essential for postnatal survival and late tooth development. In Sp3 *null* mice, the dentin/enamel layer of the developing teeth is impaired reflected by the lack of the ameloblast-specific gene products amelogenin and ameloblastin (Bouwman et al.,

2000). The analyses described here show that, in addition, Sp3 plays an important role in late bone formation. Skeletal ossification in E18.5 Sp3−/− embryos is significantly reduced compared to wild type littermates.

During embryonic development, bone formation can occur through two distinct pathways, endochondral and intramembranous ossification (Ducy et al., 2000). In the endochondral ossification pathway a cartilage template surrounded by a bone collar prefigures each future bone, whereas intramembranous ossification does not include any cartilaginous templates. In the latter case, mesenchymal precursor cells differentiate directly into osteoblasts. Our analyses of the skeleton suggest that both ways of bone formation are affected in Sp3-deficient mice. The delayed ossification of different skull bones like the parietal, frontal,
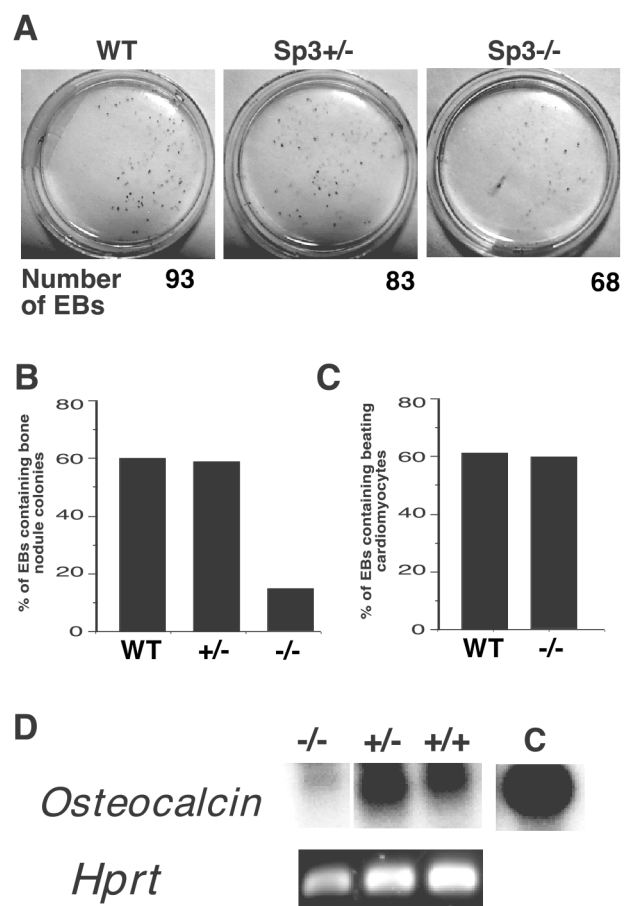

Fig. 5. Differentiation of embryoid bodies (EBs) into osteoblasts and cardiomyocytes. (A) EBs generated from wild type Sp3 + /− or Sp3−/− ES cells were treated with retinoic acid for 3 days and maintained in osteogenic differentiation medium for 15 days. Culture dishes with mineralization nodules visualized by von Kossa staining (dark spots) are shown. (B) Histogram showing the percentage of EBs containing mineralized nodules. (C) Histogram showing the percentage of EBs that contain spontaneously beating cardiomyocytes after plating. The values in (B) and (C) represent the average of two independent determinations. (D) Semi-quantitative RT-PCR with RNA of differentiated wild type (+/+), Sp3 heterozygous (+/−) and Sp3-deficient (−/−) ES cells using *osteocalcin*- or *Hprt*-specific primers. RNA prepared from MC3T3-E1 osteoblasts was used as a positive control (C).

interparietal, nasal and supraoccipital bones refers to delayed intramembranous ossification. Ossification abnormalities of the sternebrae, xiphoid process and the vertebral body are due to a delay in endochondral ossification.

3.2. Osteocalcin expression is impaired in Sp3−/− mice

We found that *osteocalcin* expression is strongly reduced in Sp3−/− E18.5 embryos. The cell that expresses *osteocalcin* is the osteoblast. Osteoblasts synthesize and secrete several extracellular matrix proteins such as fibronectin, type I collagen, osteopontin, osteocalcin and bone sialoprotein. However, osteocalcin is the only matrix protein that is specific for terminally differentiated osteoblasts. *Osteocalcin* message is expressed just after the onset of bone matrix formation (Cowles et al., 1998). Several potential functions have been attributed to osteocalcin. Its appearance may be a signal to initiate bone turnover, it may facilitate the differentiation of osteoclasts (Glowacki et al., 1991; Liggett et al., 1994) and may function as a negative regulator during the strictly coordinated processes of resorption and formation of new bone by osteoclasts and osteoblasts. Mice mutated in the *osteocalcin* gene have normal bones at birth but increased bone formation at 6 months (Ducy et al., 1996; Wolf, 1996). In E18.5 Sp3−/− embryos, however, impaired bone formation correlates with reduced expression of the *osteocalcin* gene in E18.5 Sp3-deficient embryos. Probably, the *osteocalcin* gene is not a direct target of Sp3. More likely, reduced expression of *osteocalcin* mRNA reflects a time delay of the differentiation of mesenchymal progenitor cells to mature osteoblasts resulting in a reduced number of mature osteoblasts in E18.5 Sp3−/− embryos. In that scenario, Sp3 would contribute to the process of timely coordinated osteoblast differentiation. This interpretation is supported by the in vitro differentiation experiments that show a strongly decreased potential of Sp3-deficient EB to undergo osteoblast differentiation accompanied by reduced expression of the *osteocalcin* gene.

3.3. The role of Sp3 during osteoblast differentiation

The transcription factor Cbfa1 is a key factor in the transcriptional control of osteoblast differentiation. Cbfa1-deficient mice develop a skeleton that is made exclusively of cartilage and osteoblast differentiation never occurs in these mice (Komori et al., 1997; Otto et al., 1997). In addition, Cbfa1 is a direct regulator of the osteocalcin gene in terminally differentiated osteoblasts (Ducy and Karsenty, 1995; Ducy et al., 1997, 1999; Geoffroy et al., 1995; Karsenty et al., 1999). In Sp3 *null* mice, however, expression of the *Cbfa1* gene appears not to be affected at any developmental stage investigated (E11.5, E16.5 and newborn mice). However, a minor reduction of *Cbfa1* expression cannot be completely ruled out. Haploinsufficiency of the normal *Cbfa1* product already leads to severe skeleton defects

including hypoplastic clavicles and delayed ossification of cranial bones (Otto et al., 1997).

On the other hand, it has been reported that while the amount of *Cbfa1* mRNA remains constant during osteoblastic differentiation in vitro, the binding activity of the Cbfa1 protein to DNA increases (Xiao et al., 1998). In addition, *osteocalcin* expression is not strictly dependent on Cbfa1. Calvaria-derived cells isolated from Cbfa1-deficient E18.5 embryos lacking any Cbfa1 activity are able to synthesize osteocalcin in the presence of high doses of recombinant hBMP-2 (Komori et al., 1997).

Interestingly, there might be also a joint role of Cbfa1 and Sp3 in late tooth development. Incisors of Cbfa1−/− mice show abnormal odontoblasts, dentin formation and lack enamel (D'Souza et al., 1999). In Sp3−/− newborn mice the dentin/enamel layer is also impaired due to the lack of the enamel matrix proteins ameloblastin and amelogenin (Bouwman et al., 2000).

Evidence exists that osteoblast differentiation can also be controlled in a Cbfa1-independent manner. One of the transcription factors that might be involved in this process is a homeobox protein homologous to the *Drosophila distalless* gene *Dlx5*. The skeleton phenotype of Dlx5-deficient mice resembles to some extent that seen in Sp3−/− mice. They show a delayed ossification of the membranous bones and a milder delay in bone formation in the long bones. Nevertheless, *Cbfa1* expression is not affected (Acampora et al., 1999). Like Dlx5, Sp3 could act either downstream of Cbfa1 or in a separate regulatory pathway.

4. Experimental procedures

4.1. Northern blot analyses

Total RNA from embryonic mouse tissue was extracted by the guanidinium/isothiocyanate procedure using the Qiagen kit. RNA was separated through 0.8% agarose gels containing 2.2 M formaldehyde and blotted to nylon membranes. Pre-hybridization and hybridization were carried out as described (Braun and Suske, 1998). Gene-specific probes were obtained from appropriate primer sets. Detailed information is available upon request.

4.2. RT-PCR analysis

RNA (500 ng) was reverse transcribed and cDNA amplified using the one-step RT-PCR kit (Qiagen, France). RT reactions were denatured and amplified by 32 (*osteocalcin*) or 26 cycles (*Hprt*). Primer sequences were as indicated for *osteocalcin* (5′-TCTGCTCACTCTGCTGAC-3′ and 5′-GGAGCTGCTGTGACATCC-3′), and *Hprt* (5′-GCTCC-ATTCGGATGAGTCTG-3′ and 5′-CACAGGACTAGAA-CACCTGC-3′). PCR conditions were 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C. PCR products were analyzed on 1.5% agarose gels,

blotted onto hybond membrane and hybridized with a osteo-calcin-specific probe.

4.3. Skeletal analyses

Animals were mated overnight and females examined for vaginal plug the next morning. Noon of the day of vaginal plug appearance was considered as day 0.5 post-coitum (embryonic day E0.5). For staining of cartilage and bone, animals were skinned, eviscerated and the carcasses stained with Alcian Blue (cartilage) and Alizarin Red (bone) using standard procedures (Hogan et al., 1994).

4.4. In vitro differentiation of ES cells

ES cells were maintained in Glasgow MEM/BHK21 containing 0.23% sodium bicarbonate, 1× MEM essential amino acids, 2 mM glutamine, 1 mM pyruvate, 10^{-4} M β-mercaptoethanol, 10% FBS (ES cell qualified) and leukemia inhibitor factor LIF (1000 U/ml). To induce differentiation, EB were allowed to form (Dani et al., 1997). For differentiation into osteoblasts, EBs were treated with retinoic acid (10^{-7} M) from day 2 to 5 after EB formation, then plated and maintained in osteogenic differentiation medium (50 μM ascorbic acid phosphate, 10 μM β-glycerophosphate and 2.5 μM compactin (Sigma-Aldrich, France)) for 15 days. The number of mineralization nodules was determined by von Kossa staining. Expression of *osteocalcin* was analyzed by semi-quantitative RT-PCR.

Acknowledgements

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