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The *bcl-2* knockout mouse exhibits marked changes in osteoblast phenotype and collagen deposition in bone as well as a mild growth plate phenotype

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Summary. Histological examination of long bones from 1-day-old *bcl-2* knockout and age-matched control mice revealed no obvious differences in length of bone, growth plate architecture or stage of endochondral ossification. In 35-day-old *bcl-2* knockout mice that are growth retarded or 'dwarfed', the proliferative zone of the growth plate appeared slightly thinner and the secondary centres of ossification less well developed than their age-matched wild-type controls. The most marked histological effects of *bcl-2* ablation were on osteoblasts and bone. 35-day-old knockout mouse bones exhibited far greater numbers of osteoblasts than controls and the osteoblasts had a cuboidal phenotype in comparison with the normal flattened cell appearance. In addition, the collagen deposited by the osteoblasts in the *bcl-2* knockout mouse bone was disorganized in comparison with control tissue and had a pseudo-woven appearance. The results suggest an important role for Bcl-2 in controlling osteoblast phenotype and bone deposition *in vivo*.

Keywords: Bcl-2, *bcl-2* knockout mouse, growth plate, osteoblast, bone, collagen.

During development, bone forms by two separate pathways, intramembranous- and endochrondral-ossification (IO and EO, respectively). IO is responsible for the formation of flat bones of the skull and does not involve a cartilaginous intermediate. EO is responsible for the development and growth of most other bones and involves the deposition of a cartilaginous template that is subsequently mineralized and then replaced by bone.

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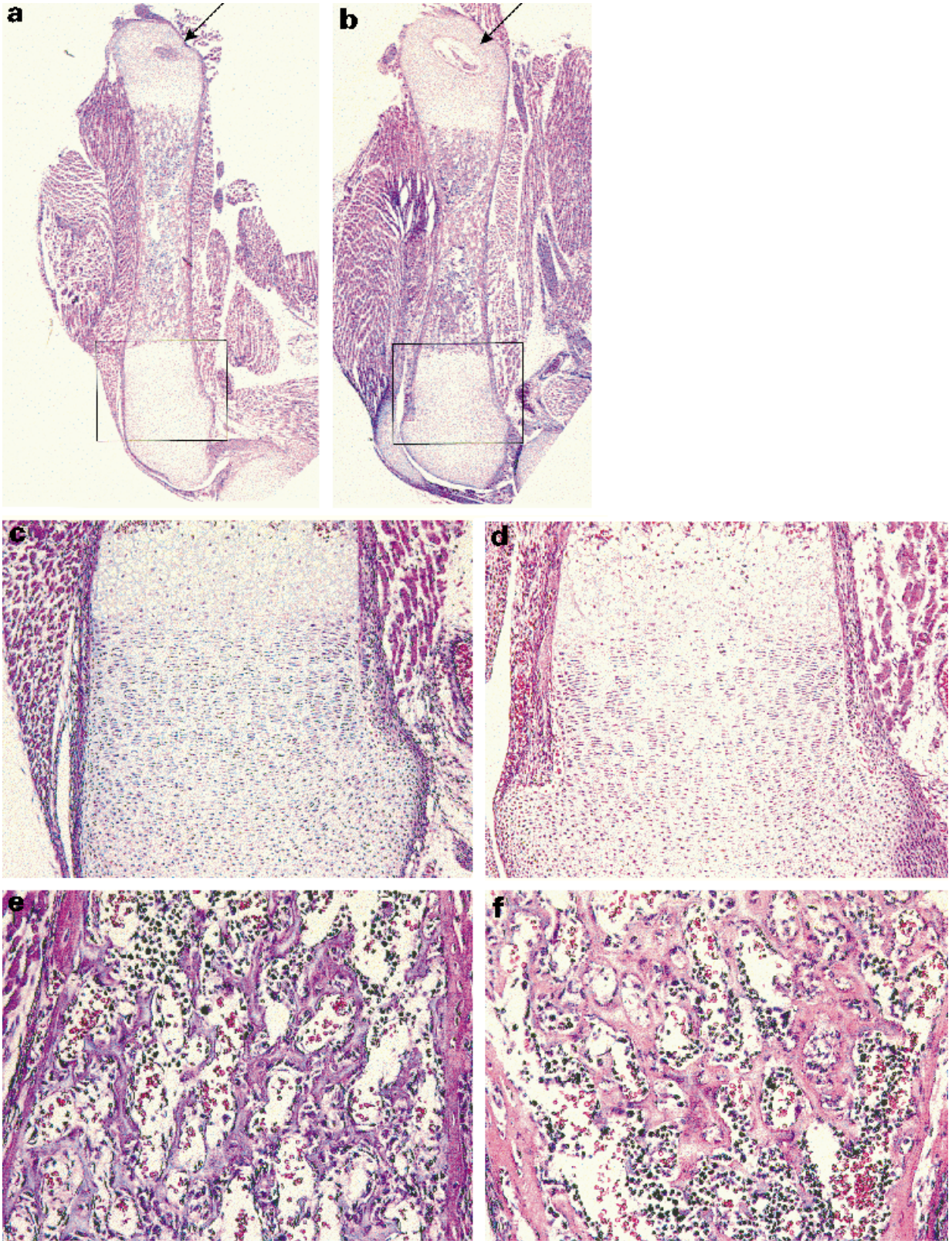
EO is initiated when mesenchymal cells condense and differentiate to form chondrocytes. These cells synthesize a framework of cartilage matrix in the approximate shape of future bones known as the anlagen. Chondrocytes in the centre of the anlagen proceed through a series of discrete developmental stages that include proliferation, maturation and hypertrophy (Poole 1991). The hypertrophic cartilage is first calcified and then, following vascular invasion, replaced by primary bone that is subsequently remodeled to form secondary bone and bone marrow. This process radiates outwards from the centre of the anlagen with the development of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structurally highly ordered growth plates that separate the cartilaginous epiphyses from the bony diaphysis. Later in development, secondary centres of ossification develop within the epiphyses.

EO involves a series of precisely timed and spatially organized events leading to the correct cellular differentiation in the growth plate; synthesis of the appropriate matrix components; formation of a scaffold for deposition of mineral within the matrix; production of catabolic factors for removal and remodelling of the matrix and vascular invasion accompanied by the recruitment of osteoprogenitor cells and osteoclasts. The recent identification of gene defects responsible for skeletal abnormalities in mouse and man have shown that not only structural components of the extracellular matrix, but also growth and differentiation factors, signalling molecules and transcription factors are important for proper morphogenesis during EO (reviewed by Erlebacher *et al*. 1995)

Using a subtractive hybridization procedure we have previously identified *bcl-2*, a proto-oncogene (Tsujimoto *et al*. 1984) and key regulator of apoptosis (Vaux *et al*. 1988) as a gene that is expressed at high levels in the growth plate compared to epiphyseal cartilage (Hillarby *et al*. 1996 & unpublished observations). The Bcl-2 protein promotes cell survival by protecting against diverse cytotoxic insults (see Yang & Korsmeyer 1996). The affects of Bcl-2 are antagonized by dimerization with genetically-related proteins such as Bax (see Huang *et al*. 1997). Amling *et al*. (1997) have recently demonstrated that Bcl-2 is expressed at highest levels in late proliferative and maturing chondrocytes, gradually decreasing as the chondrocytes hypertrophy. In contrast, Bax expression gradually increases as chondrocyte hypertrophy develops and reaches maximal levels in the terminal hypertrophic chondrocytes which appear to die by apoptosis. Little is known about the role of apoptosis in the regulation of bone deposition by osteoblasts although both *in vitro* and *in vivo* studies have shown that these cells also express Bcl-2 (Wang *et al*, 1997; Lynch *et al*. 1998).

Gene targeting experiments in mice have produced major advances in our understanding of the EO process. Of particular note has been the discovery of the role of PTHrP and its receptor in controlling the rate of growth plate chondrocyte maturation and hypertrophy, the role of indian hedgehog in regulating PTHrP expression and the demonstration that Bcl-2 lies downstream of PTHrP in a signalling pathway controlling EO (Amizuka *et al*. 1994; Karaplis *et al*. 1994; Lanske *et al*. 1996; Vortkamp *et al*. 1996; Amling *et al*. 1997; Schipani *et al*. 1997). The *bcl-*2 knockout mouse suffers from lymphocytopenia, polycystic kidney disease and of particular interest, the mouse becomes growth retarded or 'dwarfed' after birth (Veis *et al*. 1993; Nakayama *et al*. 1994; Michaelidis *et al*. 1996). In order to determine whether the dwarfing of the mouse is caused by a growth plate disturbance due to the absence of *bcl-2* expression, we have examined the long bones from *bcl-2* knockout mice. We report here that the growth plates of 35 day old, dwarfed knockout mice are only marginally affected and the major effects appear to be an osteoblast cell numbers and phenotype, and on the organization of the collagen deposited in the bone.

Methods

The creation of the *bcl-2* knockout mouse and the genotyping of offspring has been described in detail previously (Michaelidis *et al*. 1996). Mice (1 day: 4 wild type [*bcl-2* +/+]; 4 heterozygote [*bcl-2* +/−]; 2 knockout [*bcl-2* −/−]; 35 day: 4 wild type [*bcl-2* +/+]; 2 heterozygote [*bcl-2* +/−]; 3 knockout [*bcl-2* −/−]) were sacrificed by cervical dislocation and limbs fixed in 10% neutral buffered formalin for 24 hs. The tissue was maintained in 0.65 M EDTA, (pH 7.2) until radiologically decalcified (10–14 days). Following decalcification, the tissue was processed routinely into paraffin wax and longitudinal 7 μm sections mounted onto silanated slides for staining with haematoxylin and eosin. Immunolocalization of collagen type II was performed using the antibody D3 as described previously (Mo *et al*. 1994) and visualized using a secondary peroxidase conjugated antibody. Slides were examined on a Leica DMRB microscope with attached colour video camera. The images were processed on a Leica Q600 image analysis system.

Results

We have studied several of the major long bones

Figure 1. Femurs of a wild type (a) and a *bcl-2* knockout (b) mice at 1 day of age are the same length and both have a secondary centre of ossification (arrow) developing in the femoral head (magnification ×12.5). The boxed areas in (a) and (b) are enlarged to reveal the distal growth plates of the femur in the wild type (c) and *bcl-2* knockout mouse (d) which exhibit similar depths of proliferative and hypertrophic zones (magnification ×34). Trabecular bone formation in the wild type (e) and *bcl-2* knockout (f) mice are indistinguishable with the trabecular surfaces occupied by a large number of osteoblasts (magnification ×68).

(tibia, fibular and femur) in the *bcl-2* knockout mouse in day 1 and day 35 animals. In 1-day-old *bcl-2* knockout animals there is no evidence of growth retardation (see Michaelidis *et al*. 1996) and the lengths of the long bones are similar to that of age-matched wild-type controls (e.g. femur, see Figure 1a, b). The stage of bone development in the wild type and knockout mice appears identical with secondary centres just starting to form in the femoral heads of both types of mice (Figure 1a, b). Both types of mice also have similar sized proliferative and hypertrophic zones in the growth plate (Figure 1c, d). In addition, trabecular bone formation in the subchondral region appears identical between wild type and knockout mice (Figure 1e, f). At 35 days after birth, the *bcl-2* knockout mice are significantly lighter than their age-matched controls (Michaelidis *et al*. 1996). The pattern of trabecular bone forming in the secondary centres of ossification is not as intricate or well developed in *bcl-del2* knockout *vs*. wild type mice (Figure 2a, b). The structure of the growth plate of the knockout mouse is similar to that of the wild type although the proliferative zone appears thinner in the knockout compared to the wild type mice (Figure 2c, d). This latter difference is slight and its detection depends upon the precise plane of section through the growth plate taken for examination. The erosion of the calcified cartilage appears normal in the 35-day-old *bcl-2* knockout mice as judged by collagen II staining of the cartilage remnants in the subchondral bone (Figure 3a, b). However, an examination of the subchondral trabecular bone deposited in the two types of

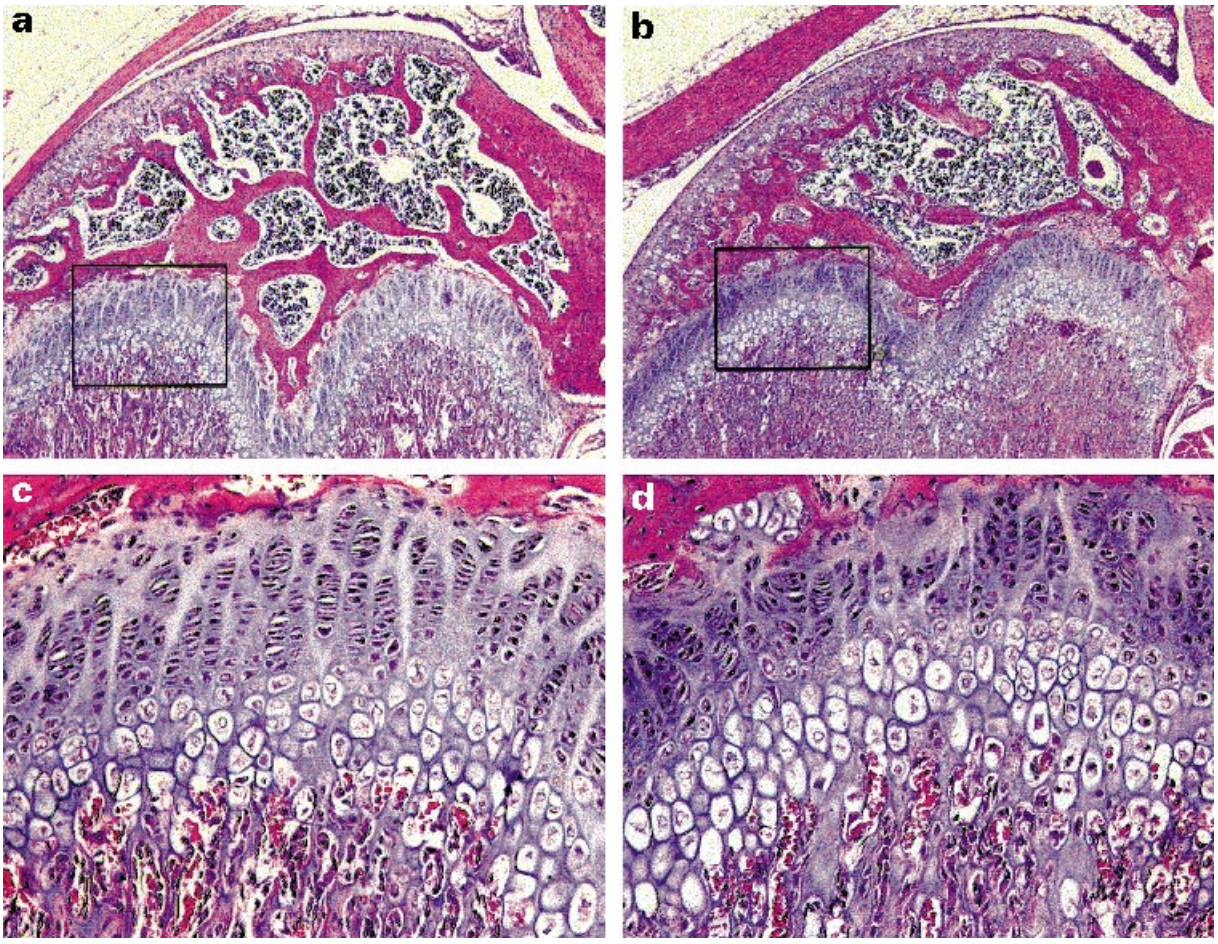

Figure 2. Trabecular bone formation in the secondary centres of ossification is reduced and less intricate in the 35-day-old *bcl-2* knockout (b) compared to the age-matched wild type (a) mouse (magnification ×40). The boxed areas in (a) and (b) are enlarged to reveal the organization of the primary growth plates in the wild type (c) and *bcl-2* knockout (d) mice. Close comparison of the two growth plates suggests that in the *bcl-2* knockout mouse (d) the proliferative zone is slightly thinner than the equivalent region in the growth plate of the wild type mouse (c) (magnification ×160).

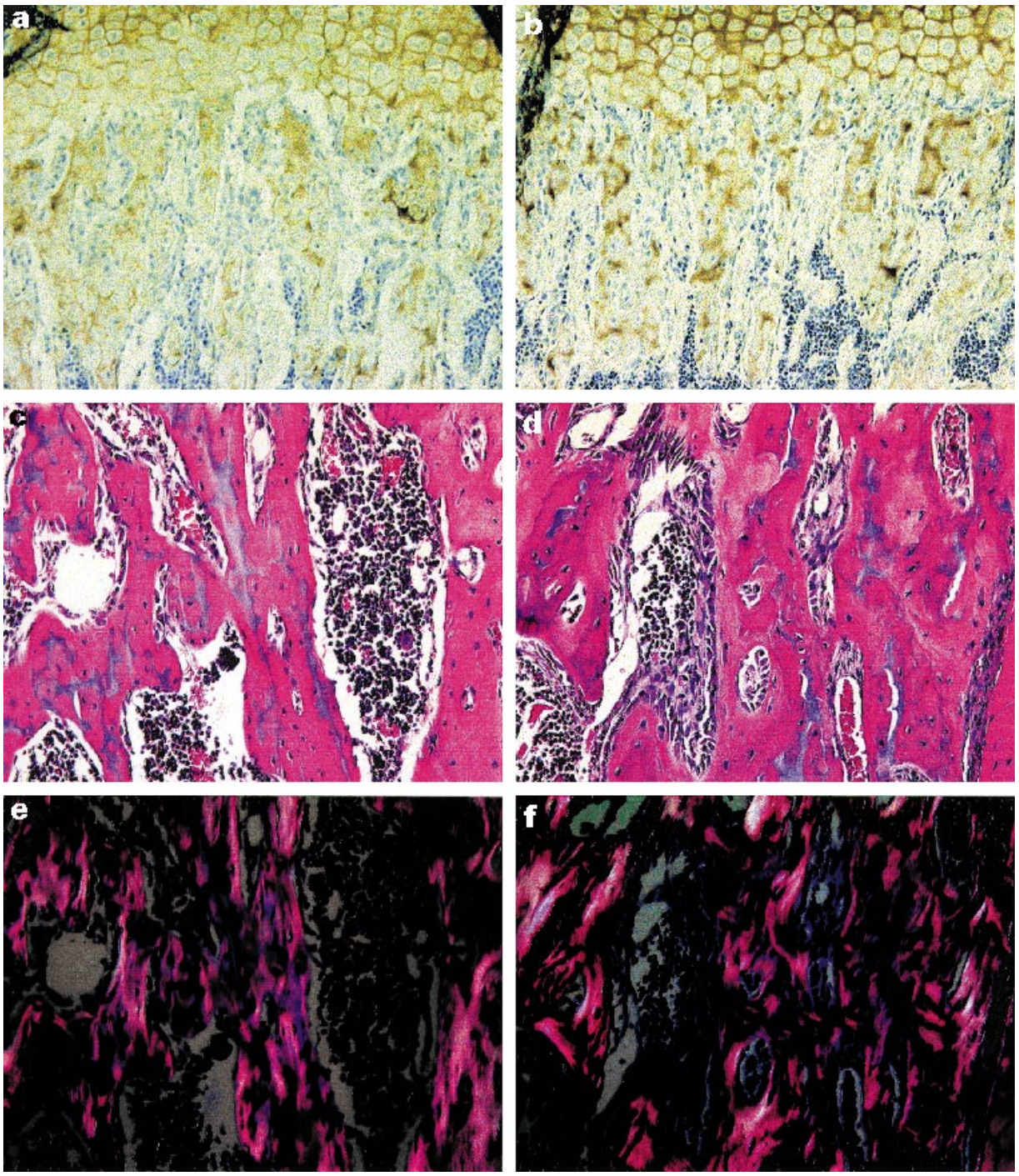

Figure 3. Immunoperoxidase localization of type II collagen (brown staining) representing the cartilage remnants in the subchondral bone of wild type (a) and *bcl-2* knockout (b) mice. The distribution of collagen II in this region is similar in both types of mouse suggesting no radical differences in the turnover of mineralized cartilage between knockout and wild type mice. Closer examination of the trabecular bone reveals the presence of greatly increased numbers of cuboidal osteoblasts occupying almost every free osteoid surface in the *bcl-2* knockout mouse (d) in comparison with similar regions in the wild type and (c) (magnification $\times$160). Viewing sections c and d under plane polarized light reveals the disorganized and pseudo-woven appearance of the collagen (brightly lit material) deposited in the *bcl-2* knockout (f) in comparison with the wild type mouse (e) (magnification $\times$160).

35-day old mice reveals striking phenotypic differences in the osteoblast populations. Normal osteoblasts in this region appear as fairly sparsely distributed flattened cells attached to the osteoid surfaces (Figure 3c) whereas in the *bcl-2* knockout mouse, the osteoblasts are far more numerous (2–3 fold increase in numbers) and appear cuboidal (Figure 3d). Viewing the tissue sections under plane polarised light reveals the organization of the collagen fibrils and fibres. The wild-type mice exhibit an ordered array of collagen fibres orientated in the longitudinal axis of the bone (Figure 3e). In comparison, the collagen fibres in the *bcl-2* knockout mouse bone are not orientated in the longitudinal axis of the bone and are disorganized with a pseudo-woven appearance (Figure 3f).

Discussion

The expression pattern of *bcl-2* through the growth plate (Amling *et al*. 1997) together with the observation that the *bcl-2* knockout mouse is growth retarded or 'dwarfed' (Michaelidis *et al*. 1996) is consistent with this gene product playing a major role in regulating the EO process. One function of Bcl-2 is to promote cell survival by preventing apoptosis. The predictable consequence of the absence of *bcl-2* expression in hypertrophic chondrocytes would be their premature demise by apoptosis due to normal increase in *bax* expression which is unaffected in the *bcl-2* knockout mouse (Amling *et al*. 1997), and a resultant decrease in growth rate. Amling *et al*. (1997) have produced evidence that EO is accelerated in *bcl-2* knockout mice due to the premature loss of terminal hypertrophic chondrocytes in the growth plate. Specifically, they have shown (i) that vascular invasion of the foot middle phalanx has occurred in 1-day knockout mice but not in age-matched controls; and (ii) that in the metatarsal bones of 6-day-old animals, the growth plate is reduced in thickness or completely replaced by bone in the knockout mouse. However, we now demonstrate that these features symptomatic of accelerated EO are not generalized features seen throughout the skeleton of the *bcl-2* knockout mouse. The 1-day-old *bcl-2* knockout mice and age-matched controls reported here, which have a similar genetic background to those reported by Amling *et al*. (1997), have long bones of equal length, indistinguishable growth plate architecture and have both just initiated the formation of secondary centres of ossification in the femoral head (Figure 1). We were unable to determine whether the slightly earlier vascularization of the foot middle phalanx in the *bcl-2* knockout mouse noted by Amling *et al*. (1997) had occurred in our mice due to the section planes used for the long bone analysis.

It is noteworthy that the effects of *bcl-2* ablation on growth rate only become overtly apparent one to two weeks after birth (see Michaelidis *et al*. 1996). Having failed to detect any significant alterations in the growth plates of the long bones of 1-day-old *bcl-2* knockout mice, we examined the same bones in 35-day-old mice where the dwarfing due to *bcl-2* ablation is marked. In these mice, there is no gross disruption apparent in the growth plates although it appears that the growth plate proliferation zones in the long bones of knockout mice are slightly thinner than in age-matched controls (Figure 2) – a similar, but far more subtle effect than that described by Amling *et al*. (1997).

Striking differences are apparent in the bone of the 35-day-old *bcl-2* knockout mice including increased osteoblast numbers, change in osteoblast cell shape and a disorganized deposition of collagen into the bone matrix. Recent *in vitro* and *in vivo* studies have demonstrated that osteoblasts express *bcl-2* (Wang *et al*. 1997; Lynch *et al*. 1998). However, it is not immediately obvious how the absence of a protein that protects against apoptosis could account for the increase in osteoblast numbers and other changes described in the bone of the knockout mouse. One possible explanation arises from recent studies describing a marked effect of Bcl-2 upon the cell cycle. These studies indicate that cells expressing Bcl-2 are more likely to exit the cell cycle and once quiescent, become refractive to mitogenic stimuli (Vairo *et al*. 1996, O'Reilly *et al*. 1996). Mutational analyses have shown that genetically distinct regions of the Bcl-2 protein are responsible for its antiapoptotic and cell cycle control functions (Huang *et al*. 1997). *In vitro* studies on osteoblast differentiation have shown that Bcl-X_L, an antiapoptotic homologue of Bcl-2, is expressed at high levels during early proliferation and differentiation and again in the fully differentiated osteocyte. However, coincident with cells exiting the cell cycle and acquiring the osteoblast phenotype, Bcl-2 expression increases and the expression of Bcl- X_L decreases (Lynch *et al*. 1998). It is possible that the up-regulation of Bcl-2 in differentiated osteoblasts functions primarily to keep the cells in G_0 and dampen their response to the mitogenic environment of the adjacent bone marrow. Accordingly, in the *bcl-2* knockout mouse, osteoblasts lacking Bcl-2 may be unable to resist re-entry into the cell cycle due to the mitogenic environment accounting for the large increase in cell numbers with downstream consequences on the cell phenotype and organization of the deposited bone matrix. It is also possible that the disorganized nature of the matrix deposited in the long bones of *bcl-2* knockout mice influences the phenotype of the osteoblasts. It is of interest to note that the

consequence of *bcl-2* ablation differs depending on the bones examined and that the phenotype is only apparent after birth (Amling *et al.* 1997; data in this paper). This is presumably due to the fact that different bones in the body develop at different times and/or rates and that the absence of Bcl-2 during EO can be compensated for in the embryo.

In summary, we have demonstrated that *bcl-2* ablation in the mouse has a marked effect on bone deposition by osteoblasts and a surprisingly mild effect on the growth plates of long bones. Further work is required to fully elucidate the role(s) of Bcl-2 in EO and osteoblasts and to determine whether the skeletal growth retardation exhibited by the *bcl-2* knockout mouse is a consequence of a defect in the growth plate or an osteoblast-based defect in bone deposition.

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