CHAPTER 6
IMMUNOLOGIC REGULATION OF BONE DEVELOPMENT

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1. ABSTRACT

A regulatory network comprised of transcription factors PU.1, Ikaros, E2A, EBF, and Pax5 control B cell fate specification and differentiation. Early B Cell Factor-1 (EBF-1) is essential for B cell fate specification while Pax5 is required for B cell development. Mice deficient in Pax5 or EBF-1 have a developmental arrest of B cell differentiation at the pro-B cell stage, which results in the absence of mature B cells. We analyzed the bone phenotype of Pax5 and EBF-1 wild-type (+/+) and homozygous mutant (−/−) mice to determine if the loss of these transcription factors regulated bone cell development.

Bones from Pax5−/− mice were strikingly osteopenic 15 days after birth, with increased numbers of osteoclasts, and decreased trabecular number. The number of osteoblasts in Pax5−/− bones and their function in vitro were not different from controls. In addition, Pax5 was not expressed by wild-type osteoblasts. To investigate the origin of the in vivo increase in osteoclasts, Pax5−/− or +/+ spleen cells were cultured with M-CSF and RANKL and multinucleated, $TRAP^+$ cells counted. Cells from Pax5−/− spleen produced 5-10 times more osteoclasts than did controls.

Tibia from EBF-1−/− mice had a striking increase in osteoblasts lining bone surfaces. Consistent with this was an increase in osteoid thickness and in the bone formation rate. This correlated with a 2-fold increase in serum osteocalcin. However, in vitro proliferation and ALP of mutant osteoblasts did not differ from control. In contrast, osteoclast number was similar in 4 week-old +/+ and −/− mice; however, at 12 weeks the number of osteoclasts was more than twice that of controls. These data correlated with an increase in bone volume at 12 weeks of age. The most striking aspect of the EBF-1−/− bones was the presence of adipocytes, which filled the marrow space. The adipocytes in the marrow were present at both 4 and 12 weeks of age. Increased fat was also seen in the liver of mutant mice. However, subcutaneous fat was almost absent in EBF-1−/− mice. Importantly, EBF-1 mRNA was expressed in wild-type osteoblasts and in adipocytes.

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Loss of EBF-1 and Pax5 causes distinct, non-overlapping bone phenotypes. It is important to understand why this network of transcription factors, which are so important for B cell development, have such striking effects on bone cell growth and development.

2. INTRODUCTION

It is clear that the immune and hematopoietic systems are closely related. In fact, immune cells arise from hematopoietic stem cells (HSC) in the bone marrow (BM). In a similar manner, the hematopoietic and skeletal systems are related. It is now clear that osteoclasts are hematopoietic in origin and are members of the macrophage lineage. However, it has only been recently appreciated that the immune and skeletal systems are also linked. Cytokines and their receptors that were thought to be expressed exclusively by immune cells have now been shown to not only be expressed by bone cells but are critical for osteoblast and osteoclast development and function. As an example, one of the ways early osteoclast precursors respond to M-CSF binding is by the expression of receptor activator of NFκB (RANK) (Hofbauer and Heufelder 2000). RANK is a member of the TNF family of receptors and is the cognate receptor for receptor activator of NFκB ligand (RANKL), which along with M-CSF, is required for osteoclast differentiation. RANK was originally identified on dendritic cells while RANKL was identified on T cells.

Osteoclast differentiation is a highly regulated process controlled by the expression of specific transcription factors and the interactions of a group of related cytokine-cytokine receptor interactions. As an example, early in osteoclast development the expression of the transcription factor PU.1 is required for further osteoclast development. PU.1 deficient mice fail to develop both macrophages and osteoclasts (Tondravi, McKercher, Anderson, et al. 1997). In addition to the requirement of PU.1 for osteoclast development, PU.1 is a member of a regulatory network of transcription factors that include PU.1, Ikaros, E2A, EBF-1, and Pax5 that control B cell fate specification and differentiation. Importantly, these transcription factors, in addition to their regulation of B cell differentiation, have important and apparently non-overlapping effects on bone cell development. This is another example of the relationship between the skeletal and immune systems and that many more unappreciated or unidentified examples await to be revealed. The study of the relationship between the skeletal and immune systems is now known as osteoimmunology.

3. EBF

EBF-1 is the founding member of small multigene family of helix-loop-helix (bHLH) proteins that are evolutionarily conserved with defined roles in cellular differentiation and function. These genes are involved in such diverse roles as sex determination in Drosophila and B-lymphopoiesis in mice (Campos-Ortega 1998; Dambly-Chaudiere and Vervoort 1998; O'Riordan and Grosschedl 1999). This motif was originally identified as a novel type of HLH protein with a dimerization domain containing two helices with homology to the second helix of the classical bHLH protein dimerization domain, but with a different type of DBA binding domain (Hagman, Belinger, Travis, et al. 1993). This factor was cloned from both S. cerevisiae in experiments aimed at identifying the olfactory-restricted olfactory marker protein-1 (OMP-1) promoter (Want and Reed 1993; Wang, Tsai, and Reed 2002) and by biochemical purification of a factor interacting with the B lymphocyte restricted mb-1 promoter (Hagman, Belanger, Travis, et al. 1993). It was named Olf-1, or Early B Cell Factor (EBF), which in turn led to the

designation of the factor as O/E-1. Mice express at least three more members of this family, EBF-2 (mMot1/O/E-3), EBF-3 (O/E2) (Walther, Guenet, Simon, et al. 1991; Dambly-Chaudiere and Vervoort, et al. 1998) and O/E-4 (Garel, Marin, Mattei, et al. 1997; Wang, Betz, and Reed 2002). Isolation of the O/E homologue Collier from Drosophila indicated the existence of a new family of evolutionarily conserved proteins [Collier/Olf/EBF (COE)].

O/E proteins play an important role in embryonic development. As an example, antisense Collier RNA results in abnormal head development in Drosophila (Crozatier, Valle, Dubois, et al. 1996). Interestingly, OAZ is an O/E interacting protein involved in BMP signaling (Hata, Seoane, Lagna, et al. 2000). OAZ interacts with Smad1 where Smads and O/E proteins compete for OAZ. Thus, it may be that O/E proteins regulate BMP signaling.

Because EBF-1 expression is critical for B cell development, mice deficient in EBF-1 lack all B cells except those at the earliest stages of development (cells expressing B220 and high levels of CD43, pro-B cells) (Hardy, Carmack, Shinton, et al. 1991; Lin and Grosschedl 1995). The absence of D-J rearrangement and the lack of Pax5 expression in EBF-1 deficient mice place EBF-1 upstream of Pax5 (Lin, et al. 1995; Urbánek, Wang, Fetka, et al. 1994). This indicates that EBF-1 is a key factor for the specification and progression of the pro-B cell after entry of the cell into the B-lymphoid pathway but before final commitment of the cell due to Pax5 expression.

In addition to pro-B cells, EBF-1 is highly expressed in adipocytes (Lin, et al. 1995). EBF-1 appears to be involved in the regulation of the glucose transporter gene *Glut4* (Dowell and Cooke 2002). All three known O/E genes are expressed in mouse adipose tissue (Akerblad, Lind, Liberg, et al. 2002). Analysis of preadipocyte cell lines (3T3 L1) indicates the genes are expressed in undifferentiated cells and expression increases with differentiation (Akerblad 2002). Over expression enhances terminal adipocyte differentiation in preadipocyte cell lines and induces adipogenesis in mutipotential cells. The fact that O/E genes are expressed throughout adipocyte differentiation raises the possibility that they are key regulators of the pathway. However, the exact mechanism of how EBF-1 stimulates adipogenesis remains to be elucidated. The role of EBF-1 in adipocyte development in vivo is also unclear. Again, the fact that EBF-1−/− mice are smaller than their WT littermates may suggest a change in the balance between osteoblasts and adipocytes, reflected in bone.

3.1. Mouse Developmemt

EBF-1 deficient mice are maintained on the C57BL/6x129 backgrounds by heterozygous matings. At birth and until 10–14 days of age the EBF-1−/− pups were indistinguishable in size from their WT littermates (visual inspection). From that point on they lagged behind their control siblings in size and growth. We have observed mice for as long as 6 months and this runting persists. The EBF-1 mutant mice had normal tooth development and their eyes were normal suggesting functional osteoclasts were present. No other visible skeletal changes were observed.

3.2. Histology-Histomorphometry

In order to evaluate bone remodeling, the femora and the tibiae from 4 week-old EBF-1−/− mice were processed for histomorphometric analysis and evaluated by light microscopy. Age and sex matched littermates were used as controls. Histomorphometric measurements were performed on a fixed region just below the growth plate, corresponding to the primary spongiosa.

In control animals the primary spongiosa contained numerous spicules extending distally from the growth plate. These spicules formed the finger-like projections of the trabeculae and the resulting bone architecture was well organized and normal. Individual trabeculae were covered with thin seams of osteoid as would be anticipated for this period of high bone formation. Cortical bone was of normal thickness and still in the process of modeling/remodeling. The growth plate was orderly with normal appearing columns of developing chondrocytes. The bone marrow was unremarkable. The secondary center of ossification was well developed with a clear demarcation line at the growth plate. Bone marrow inside the secondary center was clearly viable and the entire structure was covered with a well-developed articular cartilage.

In contrast, the bones of homozygous mutant mice exhibited profound changes. The most striking feature was the adipocytes that filled the medullary canal. Interestingly, examination of the secondary center of ossification also showed the marrow space to be filled with fat cells. The growth plates of EBF-1−/− mice were similar to controls with no obvious abnormalities. Bones from EBF-1 mutant mice were physically smaller than controls. These data indicate that the loss of EBF-1 leads to marked adipogenesis in bones with marrow (Horowitz, Bothwell, Hesslein, Plugh and Schatz 2005).

The next obvious feature was the increased osteoblasts. The number of osteoblasts was strikingly increased compared to controls. Multiple layers of osteoblasts could be seen attached to bone surfaces. The inter-trabecular spaces at the growth plate were filled with osteoblasts while individual trabeculae were surrounded by cells. In fact, almost all of the formation parameters were increased significantly. These data not only indicate a marked increase in the number of osteoblasts but also suggest that they were functional. In addition, the bone formation rate was sharply increased.

Histomorphometric analysis also indicates that the number of osteoclasts in the mutant mice was reduced from that in controls. These data, taken as a whole, indicate markedly increased bone formation with increased osteoblasts and marrow adipogenesis.

In contrast to 4 week-old mice the bone volume was now significantly increased in 12 week-old EBF-1−/− mice as compared to controls. Osteoid volume and the number of osteoblasts remained high in the EBF-1 deficient mice. Importantly, fat persisted in filling the marrow space in a pattern similar to that seen in the 4 week-old mice. These data suggest that in EBF-1−/− mice an increase in osteoblast number starts early in life and persists as young adults. However, it takes time to develop a significant increase in bone mass. These data support our hypothesis that EBF-1 regulates osteoblast differentiation.

In addition to the increase in bone, the number of osteoclasts was markedly increased in 12-week-old mutant mice as compared to controls. This increase is even more striking in light of the data from the 4 week- old mice, in which the number of osteoclasts was decreased in the mutant mice. This suggests that although the number of osteoclasts was higher, and presumably, bone resorption was also increased, the amount of bone formation exceeded the resorption.

3.3. Osteoblast Function

Although EBF-1 expression is highly restricted to the B cell lineage, adipocytes, and olfactory neurons, no data is available on its expression in bone cells. To assess EBF-1 in bone cells, WT calvarial cells were grown to pre-confluent, confluent, and post-confluent density, total RNA collected, and EBF-1 expression determined by RT-PCR. Osteocalcin and Runx2/Cbfa1 expression were also determined as additional controls. As expected Runx2 was expressed by differentiating osteoblasts and mature cells expressed osteocalcin. Importantly,

EBF-1 was expressed at all stages of osteoblast differentiation. As reported previously, and confirmed here, B cells and fat also expressed EBF-1. In contrast, no EBF-1 mRNA could be detected in mature osteoclasts.

3.4. Splenic Characteristics

Dissection of the EBF-1−/− mice revealed spleens that were by inspection substantially smaller than controls. The low cell counts are in large part due to the lack of B cells, which constitute approximately 60% of normal spleen cells.

3.5. Osteoclast Development

The histomorphometric data indicate that the number of osteoclasts is decreased in 4 week-old EBF-1−/− bone, suggesting that in vitro production of osteoclasts should also be decreased. To test this possibility BM cells from mutant or control mice were cultured with M-CSF and RANKL for 7–10 days. The cultures were then fixed and stained for TRAP and the number of osteoclasts counted. The number of osteoclasts produced from 4 week-old EBF-1−/− BM cells was reduced by approximately half compared to control. In contrast, BM cells from 12-week-old mutant mice produced similar numbers of osteoclasts as controls. These data suggest that loss of EBF-1 causes an early decrease in osteoclast progenitors that recovers with age. These data also support the histomorphometric analysis. BM is comprised of >20% B lineage cells. EBF-1−/− BM is missing the majority of these cells. This loss of B cells serves to concentrate the remaining lineages within the BM including the osteoclast precursors. Therefore, the number of osteoclasts developing from mutant BM cells should be higher than in WT BM cells. However, this appears not to be the case in 4-week-old mice. The failure of EBF-1−/− BM to produce more osteoclasts than WT further supports the idea that loss of EBF-1 effects osteoclast development.

Culture of EBF-1 deficient spleen cells with RANKL and M-CSF induced similar numbers of osteoclasts as compared to WT cells. Culture of EBF-1 deficient spleen cells repeatedly failed to produce cell lines in a manner similar to Pax5.

3.6. Fat Phenotype

Because of the unusual increase in adipocytes seen in the marrow of EBF-1−/− mice, we examined other tissues from 4-week-old mutant and control mice for fat expression. Frozen sections were cut, and stained with Oil-red-O to identify fat. No difference in Oil-red-O staining was seen in spleen, heart, or skeletal muscle . However, a striking increase in Oil-red-O staining was observed in the livers of EBF-1−/− mice as compared to control. The liver data support the idea that EBF-1 may differentially regulate fat development (deposition), in a more global manner than we originally thought.

The mice have a “skin and bones” feel and appearance, suggesting they may be cachetic. To address this possibility, full thickness skin was recovered from the dorsal surface at mid-gut from 4-week-old EBF-1−/− and WT controls. The skin was fixed and stained with H&E. A comparison of WT and mutant skin revealed a number of differences. First, the dermis of the mutant mice is substantially thinner than WT. Second, the hair follicles in the mutant skin appear very small, strikingly smaller than WT. In addition, the hair shafts are thinner in the mutants.

Third, the subcutaneous fat is dramatically reduced in the EBF-1 deficient mice, accounting for the cachetic feel of the animals. These data support our idea that the EBF-1 −/− mice are lipodystrophic.

4. Pax5

Pax5 is a member of the multigene family that encodes the paired box (Pax) transcription factors. This highly conserved motif was originally identified in Drosophila (Bopp, Burri, Baumgartner, et al. 1986). At present, nine paired box containing genes (Pax1-Pax9) have been isolated in mammals (Walther, Guenet, Simon, et al. 1991; Burri, Tromvoulis, Bopp, et al. 1989). The transcription factors encoded by the Pax genes are 128 amino acids that recognize their target genes via the DNA binding function of the paired domain (Baumgartner, Bopp, Burri, et al. 1987). These genes are involved in the regulation of pattern formation and morphogenesis because they are expressed in distinct spatially and temporally restricted patterns during embryogenesis. At present three Pax genes have been studied in mice all of which exhibit developmental mutations. The Pax1 gene is mutated in different forms of *undulated*, which exhibit skeletal changes in the vertebra (Balling, Deutsch, and Gruss 1988). Mutations of Pax3 cause *Splotch*, which causes failure to close the neural tube, absence of limb musculature, and failure to develop certain neural crest derived tissue (Epstein, Vekemans, and Gros 1991). Pax6 mutations result in the Small eye mutation, which fail to develop eyes and nose (Hill, Favor, Hogan, et al. 1991). Whether any of these mutant mice express an altered bone phenotype is unknown. Human disorders have also been associated with mutations of Pax. Pax3 is mutated in Waardenburgs syndrome, which results in deafness, and Pax6 is changed in aniridia and in Peter's anomaly (Baldwin, Hoth, Amos, et al. 1992; Ton, Hirvonen, Miwa, et al. 1991). All of these mutations suggest the importance of Pax proteins in the specialization, proliferation, and migration of progenitor cells.

The Pax5 gene codes for the transcription factor B cell lineage specific activation factor (BSAP) (Adams, Dorfler, Aguzzi, et al. 1992). BSAP is the mammalian homologue of the sea urchin protein TSAP (tissue specific activation protein). During embryogenesis Pax5 is transiently expressed in the mesencephalon and spinal code in a pattern different from other Pax genes (Adams, Dorfler, Aguzzi, et al. 1992). Later in development, expression moves to the fetal liver where it correlates with the onset of B lymphopoiesis. Within the hematopoietic system, BSAP is expressed exclusively in the B lymphocyte lineage extending from pro-B cells to mature B cells but not in terminally differentiated plasma cells (Adams, Dorfler, Aguzzi, et al. 1992; Urbánek 1994). Testis is the only other tissue in the adult mouse that expresses BSAP.

4.1. Mouse Developmemt

Pax5 deficient mice are maintained on the C57BL/6 background by heterozygous matings. At birth and until 7–9 days of age the Pax5−/− pups were indistinguishable from their WT littermates. From that point on, they lagged behind their control siblings in size and growth. At 15 days the Pax5−/− mice were severely runted being approximately 1/3 the size of the controls. Few mice survive past 17 days of age. The cause of death is unknown. The Pax5 mutant mice had normal tooth development and their eyes were normal suggesting functional osteoclasts are present. No other visible skeletal changes were observed.

4.2. Histology-Histomorphometry

In order to evaluate bone remodeling, the femora and tibiae from 15-day-old Pax5−/− mice were processed for histomorphometric analysis and evaluated by light microscopy. Age and sex matched littermates (+/− heterozygous and +/+ homozygous C57BL/6 WT) were used as controls. Histomorphometric measurements were performed as described above. In control animals, the bone architecture was well organized and normal. Individual trabeculae were covered with osteoid as would be anticipated for this period of high bone formation. Cortical bone was of normal thickness and still in the process of modeling/remodeling. The bone marrow was unremarkable.

In contrast, the bones of homozygous mutant mice exhibited profound changes. The most striking feature was the dramatic osteopenia. Overall bone volume was reduced by approximately 66% compared to controls and osteoid volume was reduced by 55%. Trabecular thickness was reduced by 18%, the number of individual trabeculae was reduced by 51%, and the space between trabeculae, another indicator of bone resorption, was reduced by 61% in Pax5 deficient mice. Observed increases in bone resorption may be accounted for, at least in part, by the >100% increase in the number of osteoclasts in Pax5−/− bone (Horowitz, Xi, Pflugh, et al. 2004). Numerous osteoclasts were observed attached to bone spicules as compared to controls. These data not only indicate a marked increase in the number of osteoclasts but also suggest that they are functional. Histomorphometric analysis also indicates that the number of osteoblasts in mutant mice was reduced from that in controls although not significantly. The bone marrow from Pax5 deficient mice appears normal with no fibrosis. These data, taken as a whole, indicate markedly decreased trabecular bone due to the large increase in osteoclasts rather than a loss of osteoblasts.

In control animals, the growth plates were robust as would be expected during this early stage of life, with orderly columns of chondrocytes. In comparison the columns of chondrocytes appear compressed in the Pax5−/− mice. Examination of the secondary center of ossification shows little bone formation with numerous chondrocytes in mutant mice versus control. The Pax5 mutant mice also lack a seam of bone separating the growth plate from the secondary center. In addition, the articular cartilage in the Pax5−/− mice is thinned and the entire structure is flattened. These data suggest a delay in development, which could account, at least in part, for the runting.

4.3. Osteoblast Function

Although Pax5 expression is highly restricted to the B cell lineage no data is available on its expression in bone cells. To assess this possibility calvarial cells (>48 hrs. old) or cells isolated from adult long bones (LBC) of WT mice were grown to confluence, total RNA collected, and Pax5 expression determined by northern blot analysis. mRNA from a transformed pro-B cell line was used as a positive control. Neither calvarial nor LBC express Pax5.

To examine the possibility that the osteoblasts from the Pax5−/− mice may be altered the proliferative and alkaline phosphatase (ALP) response was measured over time. Both the proliferative response and the production of ALP by calvarial cells were similar in Pax5−/− and WT mice.

4.4. Splenic Characteristics

Dissection of the Pax5−/− mice revealed spleens that were by inspection substantially smaller than controls. Spleen weights were taken and show that Pax5 spleens were half the

weight of WT controls. The low cell counts are in part due to the lack of B cells, which constitute approximately 60% of normal spleen cells.

4.5. Osteoclast Development

One explanation for the histomorphometric data is that the Pax5−/− mice have increased numbers of osteoclast precursors. To test this possibility spleen cells from Pax5 deficient mice and controls were cultured with M-CSF and RANKL for 7–10 days. The cultures were then fixed and stained with TRAP and the number of osteoclasts counted. Pax5 spleen cells produced more than 3 fold the number of osteoclasts than WT spleen.

Culture of Pax5−/− spleen cells for 7-10 days resulted in the outgrowth of a population of adherent cells, which could be passaged in vitro multiple times (to date 1/week for > 7 weeks). Growth of the cells did not require added growth factors. To determine whether these cells could also be induced to form osteoclasts, cells were cultured in M-CSF and RANK-L, stained with TRAP and the number of osteoclasts counted. The Pax5−/− cell line consistently produced numerous osteoclast. Interestingly, it took only 3–4 days in culture to develop osteoclasts rather than the usual 7–10 days required for unselected spleen. These data show that an unusual population of cells, enriched in osteoclast precursors, spontaneously arises from Pax5−/− spleen. No equivalent cell line could be cultured from WT spleen.

4.6. Fat Phenotype

Pax5 deficient mice do not have a fat phenotype.

5. CONCLUSION

The data show that the EBF-1 and Pax5 deficient mice are similar in that they both lack essentially all B cells except those at the very earliest stages of development and are runted shortly after birth, although the runting in the Pax5 mutants is more severe. In contrast, numerous differences exist between the mutants. The Pax5−/− mice die by 18 days while the EBF-1−/− mice can survive for at least six months. The bone phenotype of the two mutants is completely different. The EBF-1−/− mice have increased bone mass with increased numbers of osteoblasts. Interestingly, the marrow is filled with adipocytes. The Pax5−/− mice are missing two thirds of their bone mass with a marked increase in the number of osteoclasts. A continuously growing cell line with cellular and molecular characteristics of osteoclast precursors can be grown from the spleen of Pax5−/− but not from EBF-1−/− mice. These differences are more striking because EBF-1 regulates Pax5 expression, with EBF-1−/− mice being deficient in Pax5. This suggests that the EBF-1 defect overrides the Pax5 induced changes. Although B cell deficiency is common to the two mutants little evidence has been developed showing that the loss of B cells contributes to the bone phenotype. In fact, mice made deficient in B cells by other mutations (Rag and mu deficiencies) do not exhibit bone phenotypes that at all resemble the EBF-1 or Pax5 bones. However, there is a strong correlation between loss of B cells and marked changes in the skeleton. Clearly, more work must be done to determine the true relationship of this association. What is clear is that these genes were originally identified and studied because of their importance to B cell differentiation. However, as more data is developed it becomes

apparent that EBF-1 and Pax5 are central to osteoblast and osteoclast differentiation. This observation is not dissimilar to the significance of PU.1 to macrophage, osteoclast, and B cell development. The logical extension of these ideas is that other transcription factors in the hierarchy of transcription factors required for B cell development will have important effects on bone cell growth and development. Ikarous would be a likely suspect (Georgopoulos, Bigby, Want, et al. 1994). It is clear that the skeletal and immune systems share numerous factors, receptors, cytokines, and signaling pathways. These molecules are important for the interaction and regulation of these two systems. The data support the idea that other, unrecognized proteins, that will also be important to these systems await to be discovered.

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7. REFERENCES

Adams, B., P. Dorfler, A. Aguzzi, Z. Kozmik, P. Urbanek, I. Maurer-Fogy, and M. Busslinger. 1992. Pax5 encodes the transcription factor BSAP and is expressed in B-lymphocytes, the developing CNS, and adult testis. *Genes Dev* 6: 1589–1607.

Akerblad, P., U. Lind, D. Liberg, K. Bamberg, and M. Sigvardsson. 2002. Early B-cell factor (O/E-1) is a promoter of adipogenesis and involved in control of genes important for terminal adipocyte differentiation. *Mol Cell Biol* 22: 8015–8025.

Baldwin, C.T., C.F. Hoth, J.A. Amos, E.O. da-Silva, and A. Milunsky. 1992. An exonic mutation in the HuP2 paired domain gene causes Waardenburg's syndrome. *Nature* 355: 637–638.

Balling, R., U. Deutsch, and P. Gruss. 1988. Undulated, a mutation affecting the development of the mouse skeleton, has a point mutation in the paired box of Pax1. *Cell* 55: 531–535.

Baumgartner, M., D. Bopp, M. Burri, and L.M. Nol. 1987. Structure of two genes at the gooseberry locus related to the paired gene and their spatial expression during Drosophila embryogenesis. *Genes Dev* 1: 1247–1267.

Bopp, D., M. Burri, S. Baumgartner, G. Frigerio, and M. Noll. 1986. Conservation of a large protein domain in the segmentation gene paried and in functionally related genes of Drosphila. *Cell* 47: 1033–1040.

Burri, M., Y. Tromvoulis, D. Bopp, G. Frigerio, and L.M. Nol. 1989. Conservation of the paired domain in metazoans and its structure in three isolated human genes. *EMBO J* 8: 1183–1190.

Campos-Ortega, J.A. 1998. The genetics of the Drosophila achaete-scute gene complex: a historical appraisal. *Int J Dev Biol* 42: 291–297.

Crozatier, M., D. Valle, L. Dubois, S. Ibnsouda, and A. Vincent. 1996. Collier, a novel regulator of Drosophila head development, is expressed in a single mitotic domain. *Curr. Biol* 6: 707–718.

Dambly-Chaudiere, C., and M. Vervoort. 1998. The bHLH genes in neural development. *Int J Dev Biol* 42: 269–273.

Dowell, P., and D.W. Cooke. 2002. Olf-1/early B cell factor is a regulator of gult4 gene expression in 3T3-L1 adipocytes. *J Biol Chem* 277: 1712–1718.

Epstein, D.J., M. Vekemans, and P. Gros. 1991. Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax3. *Cell* 57: 767–774.

Garel, S., F. Marin, M.G. Mattei, C. Vesque, A. Vincent, and P. Charnay. 1997. Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. *Dev Dyn* 210: 191–205.

Georgopoulos, K., M. Bigby, J-H. Want, A. Molnar, P. Wu, S. Winandy, and A. Sharpe. 1994. The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 79: 143–156.

Hagman, J., C. Belanger, A. Travis, C.W. Turck, and R. Grosschedl. 1993. Cloning and functional characterization of early B-cell factor, a regulator of lymphocyte-specific gene expression. *Genes Dev* 7: 760–773.

Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution, and characterization of pro-B and Pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med* 173: 1213–1225.

Hata, A., J. Seoane, G. Lagna, E. Montalvo, A. Hemmati-Brivanlou, and J. Massague. 2000. OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* 100: 229–240.

Hill, E.R., J. Favor, B.L.M. Hogan, C.C. Ton, G.F Saunders, I.M. Hanson, J. Prosser, T. Jordan, N.D. Hastie, and V. van Heyningen. 1991. Mouse Small eye results from mutations in a paired-like homeobox-containing gene. *Nature* 354: 522–525.

Hofbauer, L.C., and A.E. Heufelder. 2000. The role of receptor activator of nuclear factor-κB ligand and osteoprotegerin in the pathogenesis and treatment of metabolic bone diseases. *J Clin Endocrinol Metab* 85(7): 2355–2363.

Horowitz, M.C., Y. Xi, D.L. Pflugh, D.G.T. Hesslein, D.G. Schatz, J.A. Lorenzo, and A.L.M. Bothwell. 2004. Pax5 deficient mice exhibit early onset osteopenia with increased osteoclast progenitors. *J Immunol* 173: 6583–6591.

Horowitz, M.C., A.L.M. Bothwell, D.G.T. Hesslein, D.L. Pflugh, and D.G. Schatz. 2005. B cells and osteoblast and osteoclast development. *Immunol Rev* 208: 141–153.

Lin, H., and R. Grosschedl. 1995. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* 376: 263–267.

O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* 11: 21–31.

Ton, C.C., H. Hirvonen, H. Miwa, M.M. Weil, P. Monaghan, T. Jordan, V. van Heyningen, N.D. Hastie, H. Meijers-Heijboer, M. Drechsler, B. Royer-Pokora, F. Collins, A. Swaroop, L.C. Strong, and G.F. Saunders. 1991. Positional cloning and characterization of a paired-box-and homeobox-containing gene from the anirida region. *Cell* 57: 1059–1074.

Tondravi, M.M., S.R. McKercher, K. Anderson, J.M. Erdmann, M. Quiroz, R. Maki, and S.L. Teitelbaum. 1997. Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* 386: 81–84.

Urbánek, P., Z-Q. Wang, I. Fetka, E.F. Wagner, and M. Busslinger. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 79: 901–912.

Walther, C., J.I. Guenet, D. Simon, U. Deutsch, B. Jostes, M.D. Goulding, D. Plachov, R. Balling, P. Gruss. 1991. Pax: A murine multigene family of paired box-containing genes. *Genomics* 11: 424–434.

Wang, M.M., and R.R. Reed. 1993. Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. *Nature* 364: 121–126.

Wang, S.S., R.Y.L. Tsai, and R.R. Reed. 1997. The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. *J Neurosci* 17: 4149–4158.

Wang, S.S., A.G. Betz, and R.R. Reed. 2002. Cloning of a novel Olf-1/EBF-like gene, O/E-4, by degenerate oligo-based direct selection. *Mol Cell Neurosci* 20: 404–414.