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Extra View

The Dissection of Human Autosomal Recessive Osteopetrosis Identifies an Osteoclast-Poor Form due to RANKL Deficiency

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ABSTRACT

Genetic dissection of human recessive osteopetroses (ARO) has identified specific subsets due to a defect in molecules linked to the effector function of mature osteoclasts. While an impairment in osteoclast differentiation in mouse leads to osteopetrosis, the four genes identified so far in classical human ARO (TCIRG1, CLCN7, OSTM1 and PLEKHM1) are all involved in the resorption and/or intracellular traffic of the minerals solubilised from bone matrix. The recent finding that the RANKL gene is mutated in a subset of ARO patients whose biopsies did not show any osteoclast shows that a differentiation defect can be responsible for human ARO and paves the way to a potential rational therapy of this rare disease by soluble RANKL administration.

INTRODUCTION

Infantile malignant Autosomal Recessive Osteopetrosis (ARO, OMIM 259700) is a severe bone disease with a fatal outcome, generally within the first decade of life.[1] A bone resorption defect, due to osteoclast malfunction, causes many clinical severe abnormalities, including: high-density ("marble") bones lacking marrow cavity, macrocephaly, progressive deafness, blindness, hepatosplenomegaly and severe anemia, beginning in early infancy or in fetal life. ARO are usually referred to as "pure" bone forms, since all these symptoms could be related to the original bone defect; deafness and blindness are generally thought to represent effects of bone pressure on cranial nerves, while anemia and hepatosplenomegaly are secondary to the lack of bone marrow space leading to extramedullary hematopoiesis.[2] However, forms associated to additional defects have also been described. Among these, those severely affecting the central nervous system (CNS) are the most difficult to evaluate, since a primary CNS defect cannot easily be discriminated from the secondary defect due to compression by abnormal skull deformities. In addition, at least two distinct entities have been clearly defined, since their underlying molecular defect has been found and shown to involve specific genes: osteopetrosis (OP) with renal tubular acidosis (OMIM 259730), which is due to a defect in Carbonic Anhydrase II and OP with anhidrotic ectodermal dysplasia and immunodeficiency (OMIM 300301), which is due to a defect in the NEMO gene. In addition, further molecular dissection of human ARO allowed us and others to show that ARO forms due to a defect in both alleles of CLCN7 or OSTM1 (grey-lethal) are indeed associated to a primary defect in the nervous system.[3,4] More recently, Van Hul's group has identified the human homologue of the gene responsible for the spontaneous *incisor absent* (*ia*) rat mutation (PLEKHM1) and shown that it was mutated in a family with an intermediate OP.[5]

The Carbonic Anhydrase deficiency was the first to be identified in humans. Interestingly this defect was not identified by genetic analysis but through biochemical studies.[6] It took 17 years from then for the first identification of the gene responsible for "pure" human ARO.[7] The possible relevance to human pathology of this gene, ATP6a3 (TCIRG1), the a3 subunit of the vacuolar proton pump, was spotted by two papers on mouse, the first showing that an artificially created *tcirg1*-deficient mouse was osteopetrotic[8] and the second demonstrating that the spontaneous osteopetrotic *oc/oc* mouse had a deletion in the 5' region of the same gene.[9] The fact that this gene mapped on the 11q13 chromosomal region, which had previously been implicated in human ARO, prompted several groups to investigate this gene in human ARO.[7,10,11]

In spite of this specific example of the relevance of mouse genetics to human pathology, it is noteworthy that several other mouse models of ARO had been described before, but

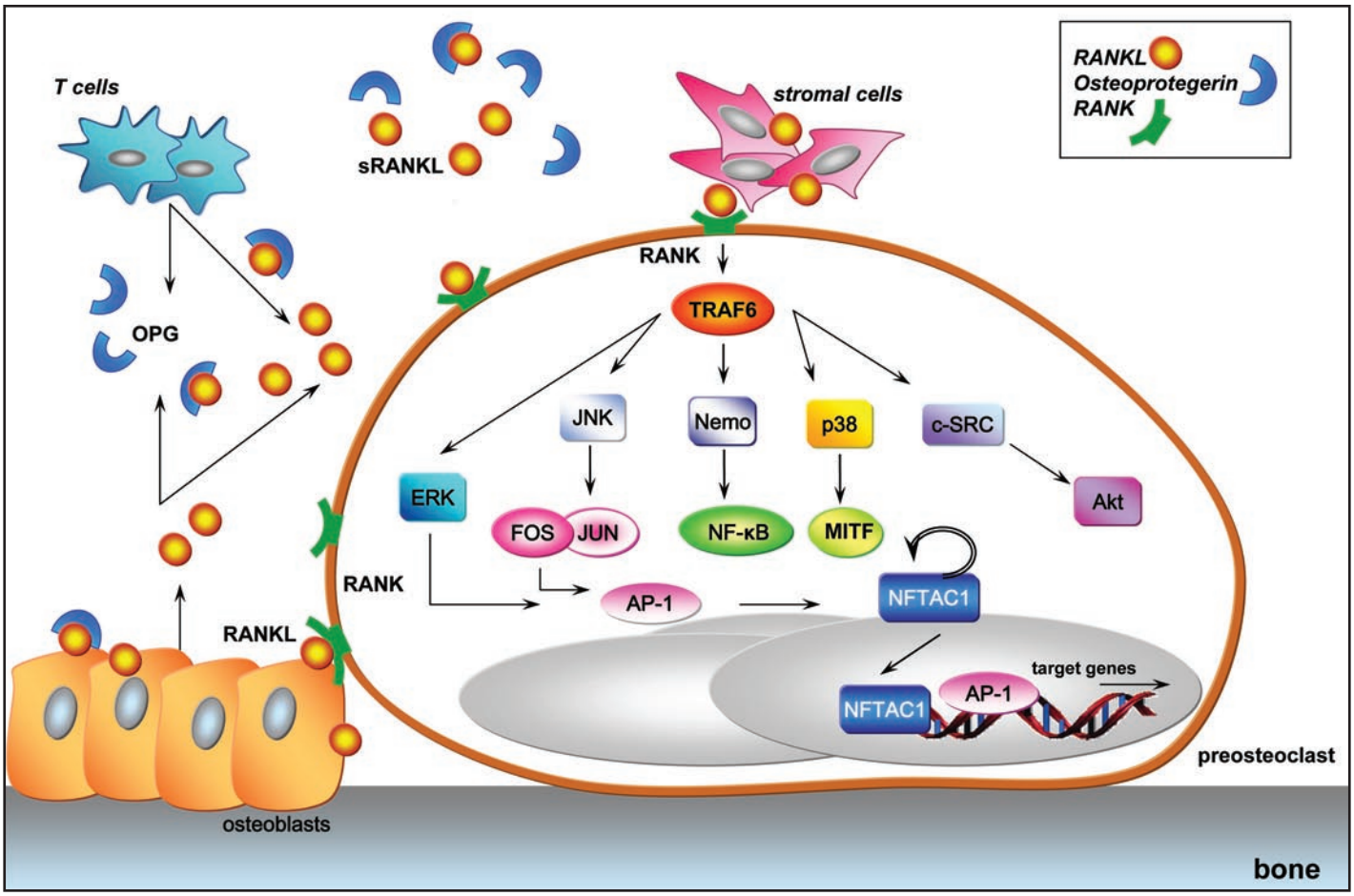

Figure 1. Schematic representation of the signaling cascade downstream RANKL/RANK binding on preosteoclasts. RANKL, produced by osteoblasts and stromal cells, interacts with its specific receptor RANK on the plasma membrane of osteoclast precursors. Consequently, signals through several pathways lead to the activation of the nuclear factor of activated T cells (NFAT) c1, which, together with AP1, drives the expression of osteoclast-specific genes, thus playing a pivotal role in osteoclastogenesis.

that investigation of these genes in humans had always been negative up to then. In addition to the spontaneous *op/op* and *mi/mi* mice, many other genes involved in osteoclast differentiation were targeted in the mouse (reviewed in ref. 12), and several of them produced an ARO phenotype.

OSTEOCLAST DIFFERENTIATION AND RANKL

Osteoclasts are multinucleated giant cells produced from hematopoietic precursors, and are responsible for bone resorption. The differentiation process requires direct interactions between osteoblasts or marrow stromal cells and osteoclasts precursors, and hematopoietic factors supplied by osteoblasts; in particular the TNF-related cytokine RANKL (also called TNFSF11, TRANCE [TNF-related activation-induced cytokine], ODF or OPGL) and the polypeptide growth factor M-CSF play a critical role (reviewed in refs. 13 and 14). M-CSF and RANKL bind to their specific receptors, c-fms and RANK respectively, expressed on osteoclast precursors, thus stimulating osteoclast formation.[15] In fact, the receptor-ligand interaction activates a signaling cascade, involving TRAF6, c-Fos and calcium signaling pathways and causing the activation of the nuclear factor of activated T cells (NFAT) c1, the master key of osteoclastogenesis[16] (Fig. 1). This ultimately results in the expression of osteoclast specific genes, such as cathepsin K (CATK), tartrate-resistant acid phosphatase (TRAP), β3 integrin and calcitonin receptor.[17] In addition, osteoblasts/stromal cells produce osteoprotegerin (OPG), a decoy receptor which inhibits RANKL function by competing with RANK for binding to RANKL.[18] Studies focused on elucidating RANK initiated signaling in osteoclasts demonstrated that RANK activates (at least) six key signalling pathways in osteoclasts: nuclear factor of activated T cell (NFAT)c1, nuclear factor kappa B (NFκB), Akt/protein kinase B (PKB), Jun N terminal kinase (JNK), extracellular signal regulated kinase (ERK) and p38.[14]

In response to all these pathways, osteoclasts differentiate and become polarized upon attachment to the bone surface (Fig. 2). The region of adhesion to bone, the sealing zone,[19] is characterized by the presence of the actin ring, made of actin filaments and specialized molecules, vinculin and talin. The space between the cells and the bone matrix constitutes the resorption lacuna. The membrane of the cells facing the resorption lacuna, the ruffled border, presents with a characteristic highly interdigitating structure.[20] Its extensive folding is probably due to the intense vesicular traffic associated with secretion of several proteolytic enzymes, such as cathepsin K and matrix metalloprotease 9, critically involved in bone mineral degradation[18] and to the need for a high number of proton pumps,

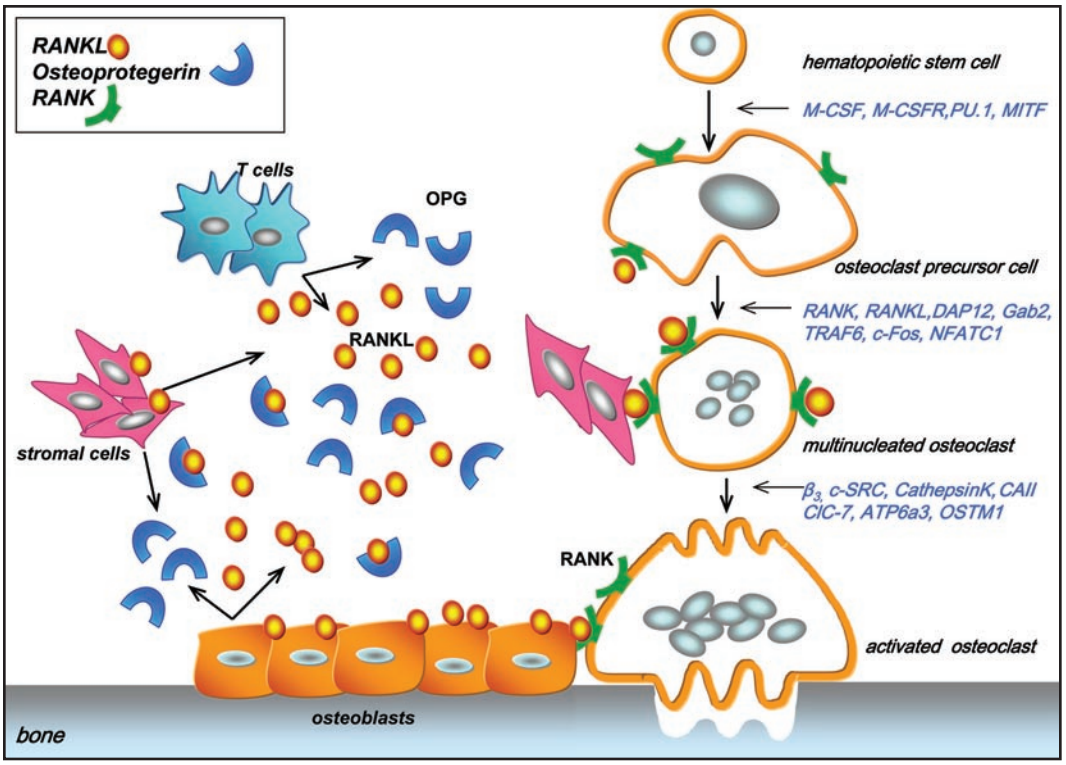

Figure 2. Schematic representation of the steps leading to the formation of mature, active osteoclasts. In the presence of M-CSF and the transcription factors PU.1 and MITF, hematopoietic stem cells give rise to osteoclast precursor cells and, after a fusion event, to multinucleated osteoclasts. On the right, the scheme shows how the disruption of genes (also listed in Table 1) in mouse leads to an osteopetrotic phenotype by impairing osteoclast differentiation at specific steps, thus giving rise to osteoclast-poor or osteoclast-rich osteopetrosis. On the left, the role of OPG, acting as a soluble decoy receptor for RANKL, thus preventing its effects on osteoclast differentiation and function, is highlighted.

which determine the low pH required for the dissolution of the mineral matrix and for the proteolytic function. The protons are produced by Carbonic Anhydrase II, which forms carbonic acid dissociating to bicarbonate and protons in the cytoplasm, and are transported into the resorption lacuna by the vacuolar proton pump (H^+-ATPase),[20,21] which is assembled from 13 different types of subunits coded by separate genes. To counteract proton extrusion by the VPP, it has been hypothesized that a chloride channel could function to dissipate the membrane potential generated. In mouse, disruption of the *clcn7* gene resulted in an osteoclast defect, leading to osteopetrosis, suggesting that ClC-7 could be the channel involved in this process.[22] However, while the role of ClC-7 in osteoclast function is fundamental, the precise mechanism of its action is still unclear.[23,24]

GENES AND OSTEOPETROSIS

Loss of function mutations in mouse have confirmed the role of many genes in the maturation of osteoclasts and highlighted their possible role in osteopetrosis. In particular, disruption of either the *rankl* gene or its receptor *rank* cause osteopetrosis in mouse.[24-27] Osteopetrosis had also been described in mice lacking the transcription factors *PU.1*,[28] *csf-1* (*op/op* mouse),[29] *c-src*[30] and *c-fos*[31,32] as well as in double knockout mice lacking both *nf-kB1* and *nf-kB2*.[33] In some of these knockouts the bone defect is associated with other abnormalities in the hematological and immune systems. All these knockout mice show lack of osteoclasts in their bones, while in other mouse models of OP osteoclasts are present, although they do not function properly (Table 1). Interestingly, in most human ARO, osteoclast number is normal or increased.[2] This finding and the fact that, in addition to TCIRG1 and CA-II, two other subsequently identified genes in human ARO (CLCN7 and OSTM1)[22,34] code for effector molecules specific for mature multinucleated osteoclasts, suggested that, in humans, the defect was not in osteoclast differentiation but most likely in genes involved in the functional capacity of mature osteoclasts.

OSTEOCLAST POOR OSTEOPETROSIS

Although it was largely assumed that human ARO was osteoclast rich, it must be pointed out that bone biopsy is only rarely performed in ARO patients for ethical reasons. When they are performed, most samples show normal or elevated numbers of osteoclasts.[7] However, a few biopsies devoid of osteoclasts were described.[35-38] Interestingly, although it is often difficult to obtain blood samples for in vitro culture from ARO patients, who are infants severely affected and rapidly transplanted, some researchers showed that, while monocytes from TCIRG1-dependent ARO patients are able to differentiate into multinucleated osteoclasts which are unable to resorb bone, those from some patients with osteoclast-negative biopsies did not.[35,36] This suggested that an osteoclast-poor ARO subset bearing defect in a gene relevant to early or intermediate stages in osteoclast differentiation could exist.

Since 2000, we have been collecting a large series of more than 200 patients with a clinical diagnosis of ARO. About 65% of patients have mutations in TCIRG1 or CLCN7,[3,39,40] while mutation in the OSTM1 gene accounts for only 1–2%[4] and the recently identified PLEKHM1 has been found mutated in only 1 patient.[5] Therefore, we carefully revised the clinical findings of patients negative for all the four mentioned genes and selected a few patients whose bone biopsy had no osteoclasts.[41] Interestingly, some of them underwent hematopoietic stem cell transplantation (HSCT) (without stromal cells) which did not improve their overall status, in spite of an apparent hematological engraftment. Although an HSCT can fail for many reasons, we hypothesized that the concomitance of this data with the absence of osteoclast could suggest a non cell-autonomous defect, such as the one that could be expected if the RANKL or the M-CSF genes were involved. Sequencing of the RANKL gene in six

Table 1 **Genes involved in the pathogenesis of osteopetrosis in mouse**

Human Gene	Mouse KO	Bone Defects	Osteoclast Defects	References
Tcirg1	*oc* $^{-/-}$	severe osteoclast-rich osteopetrosis	non-resorbing multinucleated osteoclasts	9
Clcn7	*clc-7* $^{-/-}$	severe osteoclast-rich osteopetrosis with primary CNS defects	non-resorbing multinucleated osteoclasts	22
Ostm1	*gl*$^{-/-}$*(grey-lethal)*	severe osteoclast-rich osteopetrosis with primary CNS defects	non-resorbing multinucleated osteoclasts	34
Plekhm1	*ia*$^{-/-}$ *(incisor absent rat)*	mild osteoclast-rich osteopetrosis	impaired late endosomal trafficking in multinucleated osteoclasts	5
M-CSF	*op*$^{-/-}$	mild osteoclast-poor osteopetrosis	severe deficiency in mature macrophages and osteoclasts	29
M-CSFR (fms)	*csfr1*$^{-/-}$	mild osteoclast-poor osteopetrosis	reduction in osteoclasts number	54
Cathepsin-K*	*cathepsin-K*$^{-/-}$	mild osteoclast-rich osteopetrosis	abnormal ruffled border	55
$\beta_3$integrin	$\beta_3^{-/-}$	severe osteoclast-rich osteopetrosis	failure to form actin rings or normal ruffled border	56
Rankl	*opgl*$^{-/-}$ *and trance*$^{-/-}$	osteoclast-poor osteopetrosis	no osteoclasts	26, 43
Rank	*tnfrsf11a*$^{-/-}$	osteoclast-poor osteopetrosis	no osteoclasts	27
c-Src	*src*$^{-/-}$	severe osteoclast-rich osteopetrosis	lack of ruffled border	57
c-Fos	*c-fos*$^{-/-}$	severe osteoclast-poor osteopetrosis	increased numbers of bone marrow macrophages, no osteoclasts	31, 32
Mitf	*mi*$^{-/-}$	mild osteoclast-rich osteopetrosis	small osteoclasts, no ruffled border	58
Traf6	*traf6*$^{-/-}$	severe osteoclast-rich osteopetrosis	lack in the contact with bone surfaces	59
PU.1	*PU.1*$^{-/-}$	severe osteoclast-poor osteopetrosis	no development of macrophages, no osteoclasts	28
NFκB p50/p52 NFκ p52	*NfκB1*$^{-/-}$ */ NfκB2*$^{-/-}$	mild osteoclast-poor osteopetrosis	impaired macrophage functions	33
Osteoprotegerin	*opg transgenic*	mild osteoclast-poor osteopetrosis	reduction in number of osteoclasts	60
Grb-2 associated binder 2	*gab2*$^{-/-}$	mild osteopetrosis	decreased number of osteoclast and number and average size of pits	61
Nemo#	*IKK*γ $^{-/-}$	embryonic lethal in null mice		62
Dap12	*dap12*$^{-/-}$	mild osteoclast-poor osteopetrosis	reduction of multinucleated osteoclasts	63
Dap12/FcRgamma	*dap12*$^{-/-}$ */FcRgamma*$^{-/-}$	severe osteoclast-poor osteopetrosis	impaired osteoclast differentiation	64
Vav 3	*vav3*$^{-/-}$	mild osteoclast-rich osteopetrosis	lack of actin ring	65
Syk	*syk*$^{-/-}$	mild osteoclast-rich osteopetrosis	impaired cytoskeleton of multinucleated osteoclasts actin ring	66
Pyk 2	*pyk2*$^{-/-}$	mild osteoclast-rich osteopetrosis	lack of podosomes	67
Rgs10	*rgs10*$^{-/-}$	severe osteoclast-poor osteopetrosis	impaired osteoclast differentiation	68

*Pycnodysostosis in humans. #Mutations in humans are hypomorphic.

patients showed mutations in both alleles,[41] highly suggestive for its involvement in the pathogenesis of the disease.

NORMAL DIFFERENTIATION POTENTIAL OF MONOCYTES FROM RANKL-DEFECTIVE, OSTECLAST-POOR PATIENTS

In the last few years, a reproducible assay investigating osteoclast differentiation and function has been standardized and applied to monocytes obtained from the peripheral blood of both normal and ARO patients.[42] Monocytes are purified from human peripheral blood and cultured in the presence of M-CSF and RANKL, whose combination drives precursor cells to differentiate along the osteoclast lineage axis. Cells pass through several stages recapitulating normal osteoclast differentiation, showing TRAP activity, actin ring formation, ruffled borders, multinuclearity and ability to extrude protons. Finally, multinucleated osteoclasts can be shown directly to resorb bone by plating them on dentine slices, where their activity can be judged by the width and depth of the produced pits.

In the classical TCIRG1-dependent ARO, monocytes stimulated in vitro with RANKL pass through all these stages and produce multinucleated osteoclasts which, however, are unable to produce pits. A similar defect is shown by CLCN7 patients.[35,36] On the contrary, peripheral blood monocytes from putative RANKL-mutated patients should be able, when exposed to RANKL, to complete all the maturation steps and resorb bone, since the defect is non-cell autonomous, as has been shown to occur in the mouse *rankl*$^{-/-}$ model.[26]

This indeed occurred in the three patients with RANKL mutations who were tested in this assay; the other three could not be tested since they had already been transplanted when we performed the molecular analysis.[41] Together with the genetic data, this finding strongly supports our conclusion that a subset of osteclast-poor ARO are due to a defect in RANKL production.

IMMUNE SYSTEM AND RANKL DEFICIENCY

Osteopetrotic *rankl*$^{-/-}$ mice show additional defects in the immune system, due to a defect in early differentiation of both T and B lymphocytes and show reduced cytokine production following antigen-receptor activation. In addition they lack peripheral lymph nodes but have normal splenic architecture and normal Peyer's patches.[26,43] We investigated whether RANKL-deficient patients share these defects with their rodent counterpart. For obvious ethical reasons, analysis in humans cannot be as extensive as that in mice and, fortunately, all our patients are still alive and no autoptic data is available. However, in two affected siblings we were able to analyze the immunological phenotype of peripheral blood lymphocytes with several markers such as CD3, CD4, CD8, CD19, CD45RA/RO, CD25, CD11c, CD1a, CD27, CD38, CD14 as well as the proliferation in response to various mitogens. No obvious difference was found between controls and the patients, although the existence of subtle defects could not be ruled out. However, cytokine production by T cells was analyzed in only one of the two siblings and found decreased, while investigation of dendritic cells with a set of assays did not show significant differences with normal controls. This normal immunological characterization is in keeping with the fact that all six patients were not particularly prone to infections.. Taken together, this data suggests that, at variance with mice, RANKL ablation only minimally affects the immune system. An alternative hypothesis could be that our RANKL mutations were hypomorphic, maintaining a partial function sufficient to allow normal lymph node maturation. This however looks unlikely, since in silico analysis suggested that all the mutations severely affected protein structure.

THERAPEUTIC ASPECTS

Genome studies have enormously increased our knowledge of the molecular basis of human genetic diseases. In the field of osteopetrosis, since the discovery of the role of TCIRG1 gene in human ARO, the heterogeneity of both dominant and recessive forms has been extensively dissected by genetic analysis. Two forms of autosomal dominant osteopetrosis (ADO) have been shown to depend on mutations in either the CLCN7 or LRP5 gene,[44,45] while so far at least four genes have been found mutated in ARO and another has been found in an intermediate ARO.[5,7,22,34,41] The LRP5 gene is also responsible for the osteporosis/pseudoglioma syndrome, the high bone mass phenotype, endosteal hyperostosis, craniosynostosis and exudative vitreoretinopathy;[46-49] similarly, a spectrum of phenotypes can be associated to different mutations in CLCN7 gene. These observations show that different mutations in the same gene can assign very different prognosis to the affected people.

Therefore, the individuation of the exact molecular defect not only allows a precise and early diagnosis but also identifies subsets of patients with different features, response to therapy and prognosis. In ARO, we have been able to show that the TCIRG1-dependent form is quite homogeneous in presentation, is not associated to primary defect in the CNS and responds to BMT, if a suitable donor is available. In addition, the earlier the transplant, the better the result and studies in the *oc/oc* mouse which bears the same defect, suggest that this form is potentially curable if diagnosed and transplanted in utero.[50] Biallelic abnormalities in CLCN7 and OSTM1-dependent forms, on the contrary, are associated with severe primary involvement in CNS, have a very poor prognosis and BMT is not deemed appropriate, since the CNS defects are not cured even when the hematological defect is ameliorated. In contrast, all the 6 RANKL patients described so far are still alive, although with severe stigmata, but those who have been transplanted have not responded to BMT. In this regard however, it is noteworthy that the BMT was performed with purified CD34^{+} cells or with cord blood.[41] RANKL-dependent patients are not expected to be cured by transplantation of hematopoietic stem cells alone, since although some RANKL is probably produced by T cells in specific occasions, the defect is mainly in the osteoblasts which originate from mesenchymal (stromal) stem cells (MSC). This suggests that RANKL-dependent patients could benefit from transplantation of purified (and in vitro expanded) MSCs, but this possibility must be tested in experimental models and in humans, since so far MSCs are still quite elusive. Alternatively, whole bone marrow aspirates could be used, if enough MSCs are present.

In spite of the great advances in diagnosis and characterization of genetic diseases and bone genetic pathologies in particular, molecular genetics has been quite unsuccessful in providing rational therapies. Gene therapy has only rarely been successful and, apart from BMT, cell therapy has not yet been proven very useful. In this regard, RANKL is unique among osteopetrosis since the cell defect is non-autonomous and this could pave the way to a replacement therapy which could theoretically provide a "*restitutio ad integrum*". RANKL-dependent ARO could be similar to hormone-deficient diseases which, as a group, are the most rewarding pathologies for a physician, since administration of the lacking hormone in a physiological manner usually completely rescues the defect. RANKL has a cytokine/growth factor nature, but is present in both a membrane-linked and a soluble form and it is not yet clear whether systemic injection of soluble RANKL would be able to modify the course of the disease. The situation is complicated by the existence of the soluble "decoy" receptor osteoprotegerin, although there are no data about its levels in RANKL$^{-/-}$ patients. However, studies in mouse suggest that soluble RANKL does have a clinically relevant effect, since partial rescue has been obtained in *rankl*$^{-/-}$ mice by a T- and B-cell expressed RANKL transgene[51,52] although overexpression in transgenic mice had an extreme effect on bone metabolism, leading to excessive bone resorption.[53] This aspect could probably be easily monitored in humans and should not represent a problem.

The real problem for these patients is the extreme rarity of the disease, which makes any useful drug an "orphan" drug. So far, only six patients have been described and this subset could represent about 3% of ARO, which is by itself a rare disease. In addition, RANKL is not an easy molecule to purify and huge amounts would probably be required for the duration of their life, or at least for the first years of life. However, several national and international initiatives have been launched with the aim of developing "orphan" drugs, and this possibility could be further explored.

CONCLUSION

Seven years ago, ARO was an example of a single disease with a homogeneous severe clinical picture. Now it has been subdivided in at least four different entities (not to mention the NEMO and PLEKHM1-dependent forms), which, unexpectedly, explains many of the differences once attributed to genetic background, environmental exposure (such as to infections or to medical treatment) and

secondary effects of different degree of skeleton (especially skull) deformities. Still, 30% of our patients lack a molecular diagnosis. Targeting of genes involved in osteoclast biology has identified many candidates which are worth testing. Since it is possible that these genes account for only a very small number of ARO patients, molecular diagnosis of this disease will soon become a difficult task. However, the identification of specific subsets of patients could not only be of biological significance but also have clinical relevance.

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