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TITLE: *In vivo* effects of the knockout of *Lyz2* on the process of osteoclast differentiation in mice

INTRODUCTION

The tight coupling between bone formation and bone resorption is integral to maintaining bone homeostasis [1]. If this homeostasis is knocked off balance, diseases such as osteoporosis or osteopenia can occur. Osteoporosis and osteopenia are diseases characterized by low bone mass, caused by decreased bone formation, or conversely, by increased bone resorption. Increased bone resorption occurs when there is excessive osteoclastic activity, which may in part be due to an increased number of osteoclasts. Osteoclasts are of the hematopoietic stem cell lineage, differentiating from myeloid cells such as monocytes or macrophage. Two osteoblast-derived factors are required for osteoclastogenesis: macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [2]. Once M-CSF is administered to the bone marrow precursor, it differentiates into preosteoclasts [3]. Then those preosteoclasts receive simultaneous stimulation from M-CSF and RANKL, causing them to fuse into multi-nucleated osteoclasts [3]. Osteoclasts are characterized by their multiple nuclei, ruffle borders and large number of lysosomes and by association, many lysosomal enzymes [4]. Lysosomal enzymes, such as tartrate resistant acid phosphatase (TRAP), are often used as stains since they are osteoclast markers, produced by their lysosomes. *Lyz2*, the gene for lysosome 2, is specifically expressed in myeloid cells.

In a previous study [5], interferon regulatory factor 8 (Irf8), a transcription factor that negatively regulates osteoclast differentiation, is downregulated in the transgenic mouse $Irf8^{fl/fl}; Lyz2^{Cre/+}$, where Irf8 is conditionally knocked out in myeloid cells that express *Lyz2/Lyz2-Cre*. Through this study, it is discovered that although $Irf8^{fl/fl}; Lyz2^{Cre/+}$ cultures *in vitro* exhibit a significantly increased number of osteoclasts when exposed simultaneously to M-CSF and RANKL, $Irf8^{fl/fl}; Lyz2^{Cre/+}$ mice do not develop osteoporosis like the global $Irf^{-/-}$ knockout does. These findings suggest that there are osteoclasts *in vivo* that are differentiated from myeloid cells that do not express

Lyz2/Lyz2-Cre, yielding a normal osteoclast count phenotype. This still needs to be confirmed, and research on the other possible dominant differentiation lineage of those osteoclasts needs to be done.

HYPOTHESIS

The dominant differentiation lineage progenitor of osteoclasts *in vivo* does not express *Lyz2*, where a *Lyz2* knockout will not yield an osteoporotic phenotype.

SUGGESTED EXPERIMENTAL APPROACH AND EXPECTED OUTCOMES

Aim: To confirm that there exists another possible lineage of osteoclasts where the progenitors do not express *Lyz2*, and how the lack of this gene affects osteoclasts or bone resorption.

Rationale: If the $Irf8^{fl/fl}; Lyz2^{Cre/+}$ mouse does not exhibit an osteoporotic phenotype, meaning that there is no Cre recombinase to splice out the loxP flanked gene of *Irf8*, knocking out *Lyz2* should not significantly decrease the number of osteoclasts. Osteoporotic phenotypes should not present, though other phenotypes are not out of question yet.

Generation of Transgenic C57BL/6 Mice: First, for experiment A, the same type of transgenic mice used in Saito et al.'s study will be used [5]. A $Lyz2^{Cre/+}$ mouse will be crossed with a $Irf8^{fl/fl}$ mouse, yielding a genotype of $Irf8^{fl/fl}; Lyz2^{Cre/+}$. The *Cre* transgene can be detected using the primer 5'-CTTGGGCTGCCAGAATTTCTC-3' for common, 5'-TTACAGTCGGCCAGGCTGAC-3' for wild-type and 5'-CCCAGAAATGCCAGATTACG-3' for mutant [5]. The floxed allele can be detected using 5'-TTGGGGATTTCCAGGCTGTTCTA-3' for the wild-type and 5'-CACAGGGAGTCCCTCTTACAAT-3' for the floxed [5]. Both transgenes can be verified through PCR analysis of genomic DNA extracted from the tail [6]. Upon successful integration of both transgenes, further bone analysis can be conducted. As to confirm what was stated in their study, this genotype mouse should show no significant decrease in bone mass and not exhibit any signs of osteoporosis. This will also serve as a control for the *Lyz2* knockout phenotype, along with the wild-type.

Next, for experiment B, a *Lyz2*$^{-/-}$ mouse is to be generated. In this case, the diphtheria toxin receptor-mediated cell knockout (TRECK) system, confirmed also by Saito et al. [6], will be used to specifically ablate cells that express *Lyz2*, leaving behind only the ones that do not. Cells that express *Lyz2* will express a diphtheria toxin receptor (DT-R), and will undergo apoptosis upon exposure to the diphtheria toxin (DT). The DT-R cDNA is inserted downstream of a tissue-specific promoter, and then microinjected into a fertilized egg to achieve this transgenic mouse. To verify the expression DT-R, RT-PCR analysis can be conducted to ensure that expression of DT-R is only in *Lys2* cells [7]. To confirm the successful integration of the transgene, PCR of genomic DNA from the tail can once again be performed. Additionally, immunohistochemical detection of the transgene product can be executed using anti-DT-R antibody.

Bone Analysis: For both experiments, for mice 8 to 18 weeks old, representative tibial and femoral, cortical and trabecular bone sections with von Kossa staining can be taken at 8 days and 40 days after administration of DT (50μg/kg body weight) [7]. At the same time, micro-CT images of lumbar vertebrae of each mouse can be taken, along with 3D-BV/TV measurements, structure model index (SMI) and trabecular thickness (Tb.Th) to assess the quality of the bone. 3D-BV/TV measures the ratio of bone volume against tissue volume, while SMI and Tb.Th measure strength. TRAP staining can also be performed to visualize the quantity of osteoclasts.

EXPECTED OUTCOMES

Both experiments should yield phenotypes very similar to the wild-type, developing no osteoporosis.

Experiment A: To confirm what was suggested in Saito et al.'s experiment [5], the transgenic mouse should have a similar but slightly lower osteoclast count than the wild-type, visible via TRAP staining (as the lineage that does not express *Lys2/Lys2-Cre* is thought to be dominant in vivo but not complete). All other results (3D-BV/TV, SMI, Tb.Th) should be similar but comparably increased against the wild-type due to the lower osteoclast count and therefore higher bone formation than resorption. Similar outcomes are expected for experiment B.

Experiment B: Since the hypothesis states that there exists a dominant differentiation lineage that does not express *Lys2* in vivo, the osteoclast counts (via TRAP staining) should be slightly lower in the *Lys2*$^{-/-}$ but not significantly decreased since the cells that underwent apoptosis due to DT were only partial, and not the dominant lineage. 3D-BV/TV, SMI and Tb.Th results should also be similar to wild-type phenotype and the phenotype of the transgenic mouse from experiment A. If osteoporotic phenotypes develop, then the hypothesis is proven wrong, indicating that there is another rationale for the non-osteoporotic phenotype seen in vivo in Saito et al.'s experiment instead of previously theorized [5]. If the hypothesis is proven correct, further research needs to be done to determine what the markers are for the dominant lineage, and how the lack of *Lyz2* gene affects osteoclasts and bone resorption.

Significance: These results are significant, as they yield non-osteoporotic results. Further research can be done on why this is, and perhaps whatever is uncovered can be used to rescue the osteoporotic phenotype, especially if cells of the other lineage are able to survive without the *Lyz2* gene. If the hypothesis is proven correct, and the dominant lineage does not express *Lys2*, it would be important to find out what new marker IS expressed in that lineage. Since a knockout study has now been done, an upregulation of this new marker, to incite an osteoporotic phenotype would reinforce this hypothesis.

Potential Pitfalls and Shortcomings: The largest potential pitfall of these experiments is that the two transgenic mice are very hard to generate. Sometimes the constructs do not successfully integrate, leaving only a few transgenic mice achieved. Furthermore, it was recently discovered that *Lyz2*, also known as LyzM, is no longer solely a myeloid cell marker, as it is expressed too in microglia [8]. This could potentially cause misinterpretation of the results, as well as complicate the generation of the transgenic mice for experiment B, which is dependent on the fact that expression of *Lyz2* is only in myeloid cells in the osteoclastogenesis process. Another significant shortcoming is that *Lyz2* is a gene that is expressed in myeloid cells in only mice (and *Lyz1* in mice is expressed in Paneth granule cells in the intestines). In humans, the lysozyme gene is only encoded by one single gene [8]. This could

present problems when attempting to bridge the gap between transgenic mice experiments and human clinical applications, regarding bone diseases such as osteoporosis, osteopenia or osteopetrosis.

CONCLUSION: The two proposed mouse models demonstrate the dominant lineage of osteoclastogenesis does not express *Lys2*, thereby having minimal effect on bone resorption.

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