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TITLE: *In vivo* deactivation of the Wnt signalling pathway in *Hyp* mice, a mouse model for X-linked hypophosphatemia.

INTRODUCTION

X-linked Hypophosphatemia (XLH) is a hereditary defective bone mineralization defect disease [1]. It is caused by an inactivating mutation in the phosphate regulating gene with homologies to endopeptidases on the X-chromosome (PHEX). In XLH, the body either has a decreased amount or no amount of PHEX. This, in turn, causes there to be an excess of phosphatonin fibroblast growth factor 23 (FGF23) circulating in the bloodstream. FGF23 regulates the sodium-phosphate co-transporter (NPT2) in the kidney, so its increase in circulation induces increased phosphate excretion, leading to the titular hypophosphatemia.

In XLH, it is often observed to be a high concentration of osteopontin (OPN). OPN is a highly acidic, calcium-binding, extracellular matrix phosphoprotein, secreted by bones. It is a known inhibitor of mineralization. Normally, in the wild-type phenotype, PHEX cleaves OPN at multiple sites to inactivate its mineralization-inhibiting ability, allowing for correct mineralization of the bone to occur. Without a functional PHEX in XLH cases, OPN accumulates, uncleaved, and is able to inhibit mineralization effectively, causing the mineralization defects observed in XLH bones [1]. However, the upstream regulators of OPN in bone, other than PHEX, are relatively unknown.

Like a lot of other cells, it is thought that OPN is regulated through a signalling pathway. One of the most ubiquitous pathways in the body is the Wnt/β-catenin signalling pathway. In this pathway, in the absence of Wnt (no ligand binding to Frizzled), β-catenin forms a complex with other proteins, and is ubiquinated and targeted for degradation, making it unavailable to enter the nucleus and form a complex with TCF, enabling the transcription of target genes. Conversely, when Wnt binds to its transmembrane receptor Frizzled, β-catenin does not form a complex and is free to enter the nucleus

and aid transcription of target genes. OPN is thought to be one of these target genes activated by the Wnt/β-catenin signaling pathway.

HYPOTHESIS

An osteoblast-specific knockout of *Fz* will restore the wild-type phenotype in *Hyp* mice by inactivating the Wnt/β-catenin signalling pathway and decreasing OPN accumulation.

SUGGESTED EXPERIMENTAL APPROACH

Aim: To restore the wild-type phenotype in *Hyp* mice, a model for XLH, and ultimately discover a method to treat human patients with XLH.

Rationale: In *Hyp* mice, where they possess an inactivating *PHEX* mutation, it has been observed that OPN accumulates in the cells since OPN is not cleaved and inactivated in the absence of PHEX, causing defective mineralization [1]. Activation of the Wnt/β-catenin signalling pathway has been shown to promote OPN expression in epithelial ovarian cancer cells *in vitro* [2]. It is then logically hypothesized that deactivation of the Wnt/β-catenin signalling pathway can be used to control and decrease OPN accumulation, lessening the defects in bone mineralization.

Generation of Transgenic Mice: *Hyp* mice are standardized and can be purchased, and therefore do not need to be generated from scratch. The osteoblast-specific knockout of *Fz* will be executed using the CreLoxP system. The *Cre* transgene will be expressed under an osteoblast-specific driver Osterix (Osx) to ensure tissue-specific expression. With the *Osx-Cre* driver, the Cre recombinase present in only osteoblasts will excise the floxed *Fz* gene with a LacZ stop codon, causing there to be an absence of the Frizzled receptor in the Wnt/β-catenin signalling pathway. The LacZ reporter gene will confirm successful recombination, as the stop codon before the gene will be excised with the floxed *Fz* gene. The *Cre* transgene can be detected and verified using this pair of primers: 5’ GCC TGCATTACCGGTCGATGCAACGA-3’ and 5’-GTGGCAGATGGCGCGGCAACACCCATT-3’ [3].

The floxed allele can also be verified using qRT-PCR to quantify the absence of *Fz* mRNA transcripts. The resulting transgenic mice will be *Fz$_{flox/flox}$;Osx-Cre,* then crossed with *Hyp* mice, with the final *Fz$_{flox/flox}$;Osx-Cre; Hyp* mice.

Appropriate Controls: Standard wild-type mice and *Hyp* mice will serve as controls. Mice with the administration of LiCl, a chemical Wnt/β-catenin activator, into osteoblasts will serve as a control as the *Hyp* mice phenotype [2] if possible. Mice with the administration of XAV939, a Wnt/β-catenin inhibitor, into osteoblasts will serve as a control for our *Fz$_{flox/flox}$;Osx-Cre;Hyp* mice phenotype [2] if possible. *Fz$_{flox/flox}$;Osx-Cre* mice can serve as an hypermineralization control.

Methods:

The following bone analyses will be performed on the *Fz$_{flox/flox}$;Osx-Cre;Hyp* mice to observe wild-type and mutant phenotypes: immunoblots, radiographs, microscopy, and immunohistochemical staining [1]. Immunoblots will be performed to quantify the expression of OPN in the mutant and control mice. Radiographs, microscopy and immunohistochemical staining will be performed to visualize the mineralization defects and its subsequent rescue in the mutant and control mice. Radiographs will be taken of the lower extremities of each mouse. Microscopy and immunohistochemical staining will be used to observe mineralization defects in the femur.

Potential Pitfalls and Shortcomings: The controls listed for administration of Wnt/β-catenin signalling pathway activators/inhibitors may not be realistically done in vivo, as it is difficult to restrict the administration of these substances to only osteoblasts, especially since the global administration of these chemicals will disrupt the Wnt/β-catenin signaling pathway that is used in a multitude of other cells. The Wnt/β-catenin signaling pathway is often a target in cancer therapy studies. Even if these chemicals are only injected into bone, there is no telling how it will affect osteoclasts, osteocytes, etc. Another shortcoming is as said, that the Wnt/β-catenin signalling pathway is fairly ubiquitous, in the sense that disrupting this pathway in osteoblasts may have some unpredicted repercussions besides the decrease in OPN expression. A better way to control OPN expression in *Hyp* mice would be to

determine what protein is causing Wnt to bind to Frizzled and activating the pathway instead of disrupting the entire pathway altogether. For example, it has been shown in previous studies that the expression of Capn4, a cysteine protease, upregulates the expression of OPN via the Wnt/β-catenin signalling pathway in epithelial ovarian cancer cells [2]. However, it has also been shown that a Capn4 knock-out in osteoblasts actually causes many problems with osteoblast differentiation, proliferation, and matrix mineralization [4]. Although Capn4 is the activator of the Wnt/β-catenin signalling pathway in EOC cells, that does not seem to be the case in osteoblasts, an unexpected outcome. Additionally, a constant pitfall that is always present with the Cre-LoxP system is leaky expression; not all cells that express Cre will successfully excise the floxed allele, so the use of a reporter gene such as LacZ is crucial.

EXPECTED OUTCOME AND SIGNIFICANCE

Expected Outcome: The *Fz$_{flox/flox}$;Osx-Cre;Hyp* mice (and the XAV939 administration) are expected to yield a wild-type-like phenotype in all bone analyses, while the LiCl administration should yield the same phenotypes as *Hyp* mice. However, the *Fz$_{flox/flox}$;Osx-Cre* mice are expected to have a deactivated Wnt/β-catenin signalling pathway, meaning that OPN does not accumulate and inhibit mineralization, leading to hypermineralization. Since the transgenic mouse *Fz$_{flox/flox}$;Osx-Cre;Hyp* are supposed to yield wild-type phenotypes, only the mutant phenotype of the *Hyp* control mice will be delineated in detail below. In the immunoblots, it is expected that compared to the wild-type mouse, the *Fz$_{flox/flox}$;Osx-Cre* mice should have a severely decreased amount of OPN expression, and that the *Hyp* mice should possess high expression of OPN. The ultimate transgenic mice, *Fz$_{flox/flox}$;Osx-Cre;Hyp*, should therefore have a rescue phenotype as a result. Similarly, in the radiographs of the lower extremities of *Hyp* mice, irregular growth plates and widened physes should be observed, as well as some metaphyseal bowing of the femorae and tibiae. In the microscopy observations of *Hyp* mice, hypomineralized “halos” around the osteocytes can be seen, the hallmark of XLH, for which *Hyp* is a

mouse model. In the immunohistochemical stainings, the classic hypomineralized "halos" of *Hyp* mice are stained magenta while the mineralized bone matrix is stained green, where large patches of magenta can be observed in *Hyp* mice.

Significance: It was already known that with a PHEX mutation, there is an increase in OPN, causing mineralization defects. More needs to be understood about the upstream determinants of OPN accumulation. In this case, it is hypothesized that OPN is regulated by the Wnt/β-catenin pathway, but it is still unknown what is causing Wnt to bind to Frizzled and activating it. If this mechanism could be better understood, more treatment options could be provided to choose who suffer from XLH, behind the current treatments that focus on treating the phosphate wasting, such as 1-α-hydroxylated Vitamin D analogs, phosphate supplementation and antibodies against FGF23 [1].

CONCLUSION

Hyp mice, a mouse model for XLH, should be able to be restored to wild-type phenotype by decreasing the characteristic OPN accumulation that is often associated with XLH. This is hypothesized to be done by crossing *Hyp* mice with an osteoblast-specific knock out of Frizzled known as $Fz_{flox/flox}$*;Osx-Cre*, with the resulting transgenic mice $Fz_{flox/flox}$*;Osx-Cre; Hyp*. More investigation is required to understand the full activation pathway of OPN, specifically how to decrease its build-up in the absence of PHEX, in order to open up the more possibilities of treatments for XLH patients that are not phosphate-centric therapies.

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