SIT as a Potential Bone Mass Regulator in Humans

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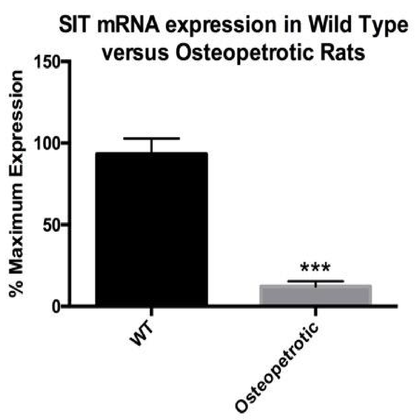
1. Introduction

SIT (SHP2-interacting transmembrane adapter/Signaling threshold regulating transmembrane adapter/Gp30/40) is a 196 amino acid glycosylated disulfide-linked homodimer that belongs to a group of transmembrane adapter proteins) that are not localized to membrane lipid rafts. Previously, SIT was thought to be exclusively expressed in T-cells where it inhibits T-cell receptor signaling. However, our lab identified SIT in osteoblasts and bones from SIT gene knockout mice display changes in many parameters of bone mass and bone formation. Likewise, osteopetrotic rats (too much bone) display decreased SIT mRNA expression compared with wild type. Previous immunohistochemical analysis also demonstrated SIT protein in osteoblasts on the endochondral surface of diaphyseal region of cortical bone. So far, all these data have been generated in rodent systems. Our goal is to begin to examine the role of SIT in human cells by reducing its expression using the crispr/Cas-9 system and examining the consequences on the expression of bone phenotype markers. Initially, we are focusing on the human osteosarcoma cell line SaOS-2 due to its ease of growth. We have successfully cloned a human SIT guide RNA into the px458 Cas-9 plasmid and are working on establishing transfection conditions by which we can introduce the plasmid into the SaOS cells. We are also working on validating RT-qPCR primer sets for human SIT and for bone phenotype marker genes including H4 histone, alkaline phosphatase, and osteocalcin in order to determine the consequences of reduced SIT expression.

2. SIT Expression and Bone Mass

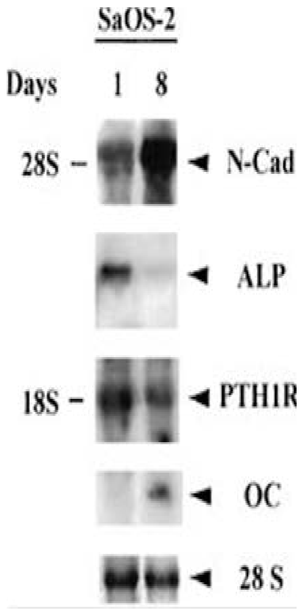


SIT was first found in bone as a gene whose expression decreased in rats with osteopetrosis as compared to their wildtype (WT) littermates. *** p<0.001.
3D reconstructions of WT (**A**) and SIT KO (**B**) right femur scans rof SIT knockout mice reveal an increased amount of trabecular bone in the SIT KO bones compared with WT bones.
In both model systems, reduced SIT levels are associated with increased bone mass further supporting the hypothesis that SIT plays a role in regulating bone cell development and function.

3. Goals

- Assay phenotypic marker genes for human SIT and other osteoblastic marker genes via RT-qPCR
- Define transfection conditions for the px458 Cas-9 plasmid into SaOS-2 cells to control the expression of SIT
- Successfully transfect the px458 Cas-9 plasmid that contains the SIT guide RNA into SaOS-2 cells
- Verify the consequences of reduced SIT expression compared with the expression of the assayed osteoblastic/phenotypic marker genes

4. SaOS-2 Development and Osteoblastic Gene Expression



SaOS-2 ("sarcoma osteogenic") is an osteosarcoma cell line derived from a primary human osteosarcoma and is the model utilized in this project. In work by Ferrari et al, N-Cad (N-cadherin) levels acted as a relative control for development in this cell line which is measured on Days 1 and 8 of culture. N-Cad showed increased expression, while ALP (alkaline phosphatase) a gene associated with increased differentiation in normal cells decreased with time. Osteocalcin, a late marker of osteoblast differentiation showed an increase in expression with time in culture.

We seek to confirm these Northern blot results with ALP and OC expression in our SaOS-2 cells in order to validate them as a potential model in which to manipulate SIT expression and assess the effects on osteoblast differentiation markers.

5. Validation of Primer Sets for Bone Phenotype Marker Genes

SaOS-2 cell RNA was isolated and reverse transcribed into cDNA for use in establishing conditions for amplification of the bone marker genes OC and ALP via PCR. To date, the results have been inconclusive despite the use of several different primer sets. As such we are in the process of acquiring human bone marrow to aid in validation of these osteoblastic marker genes. Establishment of successful PCR reaction conditions for these and other human osteoblast markers will allow to quantitatively assess the effects of either deletion or overexpression of SIT in human cells.

6. CRISPR-Cas9 Transfection

After successfully cloning a human SIT guide RNA into the px458 Cas-9 plasmid, efforts were made to establish transfection conditions for the purpose of introducing the newly created plasmid into the SaOS-2 cells. Initially it was hypothesized that the same transfection conditions which worked to introduce plasmids into ROS osteosarcoma cells would work for the human SaOS cells. Unfortunately this has proven not to be the case. When the transfection was performed under these conditions, neither the plasmid's nor the control "green lantern" GFP fluorescence could be seen in our fluorescent microscope, indicating that the transfection had not worked. After a period of experimentation we arrived at conditions that allow for transfection to occur. This involves giving each well of SaOS cells to be transfected 0.5 microliters of the px458 plasmid along with 1, 50, 2, and 25 microliters of p3000 media, Optimum media, and Lipofectmin 3000 media.

7. Conclusions and Future Studies

1. Reduced SIT mRNA expression is associated with increased bone mass in osteopetrotic rats and SIT KO mice show increased amounts of trabecular bone compared with WT bones. This suggests an association of reduced SIT levels and increased bone mass.
2. Osteosarcoma cell lines have varying osteoblastic gene expression compared to non-tumor cell lines, so we plan on using bone marrow cells as well in future experiments to validate the phenotype marker genes Alkaline Phosphatase and Osteocalcin.
3. Transfection conditions for the introduction of the SIT gRNA/px458 plasmid into the SaOS-2 cells are different from those in ROS cells and therefore a new set of transfection conditions must be worked out.
4. Our lab's previous studies suggest that SIT plays a role in regulating bone mass in rats and mice. Showing the same role for SIT in a human model of osteoblast differentiation would provide convincing evidence to the larger scientific community that this signaling pathway is important in the regulation of bone mass.

8. References and Acknowledgements

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