Development of PDIA3 and VDR Knockout Human Osteosarcoma SaOs-2 Cells Using CRISPR-Cas9

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Introduction:

Vitamin D signaling is involved in physiological calcification as well as in pathological conditions such as Fahr's disease (Keasey et al, 2016). The discovery of Vitamin D's in vitro role in calcification of human osteosarcoma SaOs-2 cells has led to further exploration of Vitamin D related signalling. The active metabolite of Vitamin D, calcitriol, exerts its intracellular effects through its interaction with protein disulfide isomerase A3 (PDIA3) at the plasma membrane. Using CRISPR-Cas9, the PDIA3 gene can be mutated in cells to knock out protein expression.This allows for in vitro cell culture manipulations to elucidate the role of calcitriol-PDIA3 signaling in calcification. To better understand the role of PDIA3 mediated vitamin D signaling in calcification, we chose to develop a cell line deficient in PDIA3 by CRISPR-Cas9. Using CHOP-CHOP, a gRNA selecting software which chooses sequences with low predicted non-specific binding probabilities, we selected gRNAs targeting exon 1 of PDIA3. The most specific gRNA was directionally cloned into a Cas9 plasmid, which along with a puromycin resistance gene plasmid, were transfected into SaOs cells. Subsequently, cells were treated with puromycin to eliminate non-transfected cells. Next, we isolated multiple homogenous populations of cells, each derived from a single transfected cell, and the expression of PDIA3 mRNA and protein was quantified for each colony. With successful confirmation of total PDIA3 knockout, further research can now begin into how calcitriol-PDIA3 signaling relates to calcification in vitro.

Methods:

Cloning: A gRNA targeting exon 1 of PDIA3 was created with the following primers: forward 5'GTGGCGCTGCTTCTTGCCG'3 and reverse complementary 5'CGGCAAGAAGCAGCGCCACA'3. For cloning of the gRNA into the CRISPR-Cas9 plasmid, a "GATCG" overhang was appended to the 5' end and "G" was appended to the 3' end of the forward strand. For the reverse strand "AAAC" was appended on the 5' end and "C" was appended to the 3' end. Restriction enzymes BamH1 and BsmB1 were used to digest the plasmid producing overhangs compatible with the annealed gRNA oligonucleotide. Ligation was carried out using standard protocol and the plasmid was amplified in DH5 E. Coli with ampicillin selection. Identity of the plasmid was confirmed by agarose gel electrophoresis.

Cell culture: SaOs cells were maintained in DMEM supplemented with 10% FBS and 2mM L-glutamine. The cells were housed in a T75 flask and were passaged once reaching 90% confluency or every 3-4 days. After passaging, 2.0E6 cells were placed back in each flask, kept at 5% CO_2 at 37°C.

qPCR:RNA was isolated using the OmegaBiotek RNEasy kit according to the manufacturer's protocol. qPCR was carried out as previously described (Keasey et al, 2013) and analyzed using the ΔΔCT method.

Western blotting: Proteins were isolated in RIPA buffer supplemented with PMSF and Western blotting was carried out as previously described (Keasey et al, 2013). Primary and secondary antibody incubation was completed in 0.5% milk in TBST. Primary antibody dilutions were: ERp57-1:1000 and GAPDH - 1:3000. The secondary antibody dilution was 1:5000.

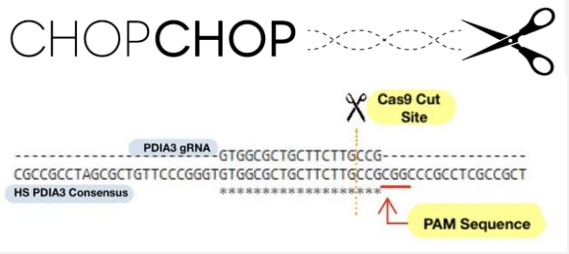

Figure 1: gRNA Selection Using CHOPCHOP Software

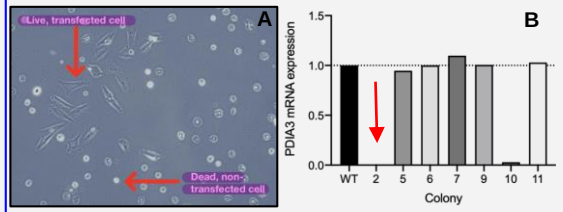

Figure 2: (A) Transfection of Cells with Cas9-gRNA plasmid and subsequent puromycin selection. (B) Single cell colonies were picked for analysis of PDIA3 mRNA expression. Here, it is seen that colonies 2 and 10 display completely knocked out PDIA3 mRNA expression. Colony 2 (red arrow) was selected for further characterization and confirmation of PDIA3 knockout.

Figure 3: Ligation of guideRNA oligonucleotides into pCas-Guide plasmid**.** (A) Restriction digest with BamHi and BsmB I was followed by sticky end (red font) ligation of gRNA oligonucleotides into pCas-Guide vectors . Diagram B depicts the mapping of the genes for the Cas9-gRNA expression plasmid.

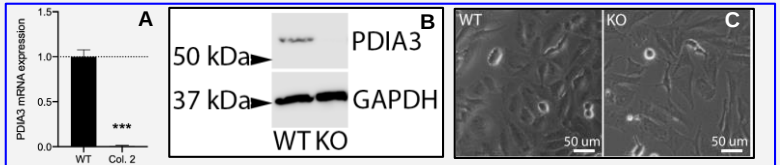

Figure 4: Colony 2 was selected for further investigation. RT-qPCR (A) and western blotting (B) confirm 100% knock out of PDIA3 in SaOs-2 cells ($p<0.001$**, Student's t-test, n=3). Brightfield images (C) demonstrate morphology of wild type (WT) and PDIA3 KO cells.

Conclusion:

- PDIA3 protein was completely knocked out for use in further analysis and experimentation on the signaling of and the role of Vitamin D in calcification processes in vitro.
- After multiple passages, morphological differences have been observed between the SaOs-2 WT and SaOs-2 PDIA3 KO cells.
- Future steps will include comparison of the ability of WT SaOs cells to undergo calcification relative to PDIA3 KO cells as well as investigation into PDIA3's role in regulating expression of mineral transporters involved in calcification.
- Though it has been reported that whole organism knock out of PDIA3 is lethal at E13.5 in mice (Coe et al, 2009), our PDIA3 KO SaOs cells successfully grow in culture.
- Apoptosis and cellular growth rates remain to be characterized.