# Effects of Phd3 Knockdown on HIF-2a in HepG2 Cells

Tavisha Thapar, Matthew Riopel, Jerry Olefsky

UCSD School of Medicine

## Abstract

Hypoxia, or a condition characterized by a deficiency in oxygen's ability to reach cell tissues, is known to increase insulin resistance in cells, contributing to diabetes. Hypoxia inducible factors (HIFs) regulate hypoxia within cells and have been implicated in insulin resistance, while prolyl hydroxylase protein isoforms (Phd1, Phd2, Phd3) degrade HIFs. It was shown that knocking out the Phd3 gene in the mouse hepatocytes stabilizes HIF-2a in murine hepatocytes, which in turn leads to increased insulin sensitivity and improvement of diabetes.

The purpose of my mentor's project is to investigate the effects of PHD modulation with respect to HIF stabilization and insulin sensitivity in human hepatocytes. My role in the project was to use a process called qRT-PCR, or real-time reverse transcriptase polymerase chain reaction, on extracts from HepG2 cells (human hepatocytes), so that it can be verified that the correct gene was knocked down, and to see if other genes are being affected by the knockdown procedure.

## The Knockdown

PHD3 was knocked down using siRNA, or silencing RNA, which is a double-stranded RNA molecule that prevents specific genes' expression by breaking down mRNA before it is synthesized into protein.

The siRNA enters the cell and is broken into fragments, one of these fragments being the antisense strand (the strand of RNA complementary to the target mRNA). A fragment (most often the antisense fragment) is loaded onto a protein called the RISC: RNA-Induced Silencing Complex. The strand bound to the RISC links to its complementary mRNA, and the mRNA is then destroyed, preventing protein synthesis from that strand of mRNA. The strand that binds to the RISC is random, which is why it is important to use qPCR to ensure that the correct gene has been knocked down.

## Methods

RNA is isolated from cells using a commercially available kit that uses a phenol-chloroform extraction. Using the enzyme reverse transcriptase, the RNA is reverse transcribed to cDNA, which is the double-stranded DNA complementary to the RNA, so that it can be more accurately measured by the qPCR machine. This cDNA is diluted, and then is mixed with primer and SYBR Green (the fluorescent dye for the qPCR). The samples are then loaded into wells and fed into a qPCR machine, which performs the PCR and produces the data. As the machine cycles, SYBR green intercalates into the PCR product, which produces a fluorescent signal for the machine to read. The raw data is provided as cycle threshold, which is then normalized using the 2Δct method. This normalizes changes in gene expression to the changes of the loading control (RP2) and the control sample.

## References

Laboratories, B.-R. (2016). *What is real-time PCR (qPCR)?* Retrieved July 26, 2016, from Bio-Rad USA, http://www.bio-rad.com/en-us/applications-technologies/what-real-time-pcr-qpcr

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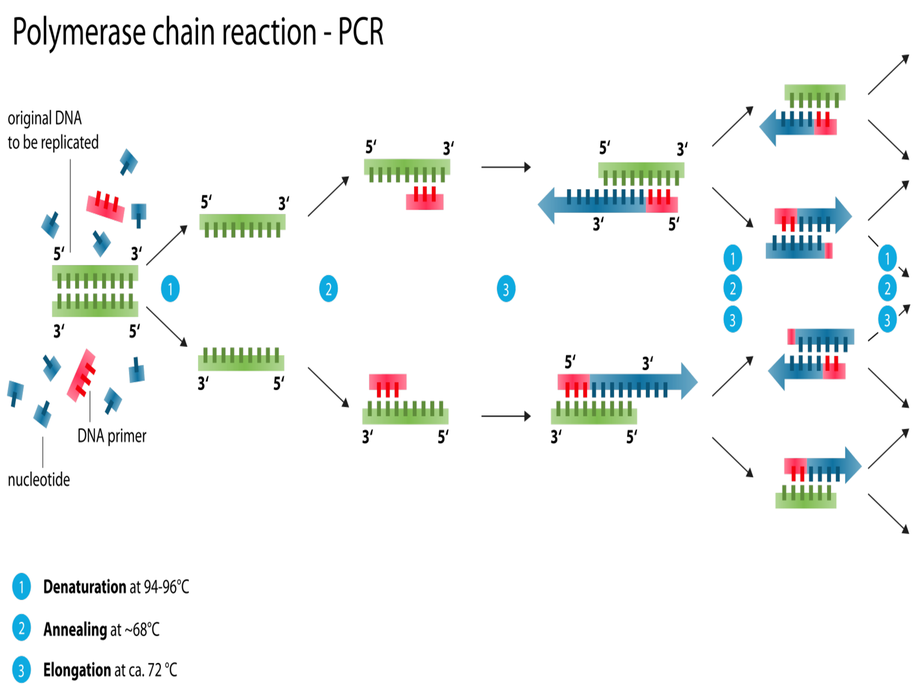
PCR Image: https://en.wikipedia.org/wiki/Polymerase_chain_reaction

## PCR



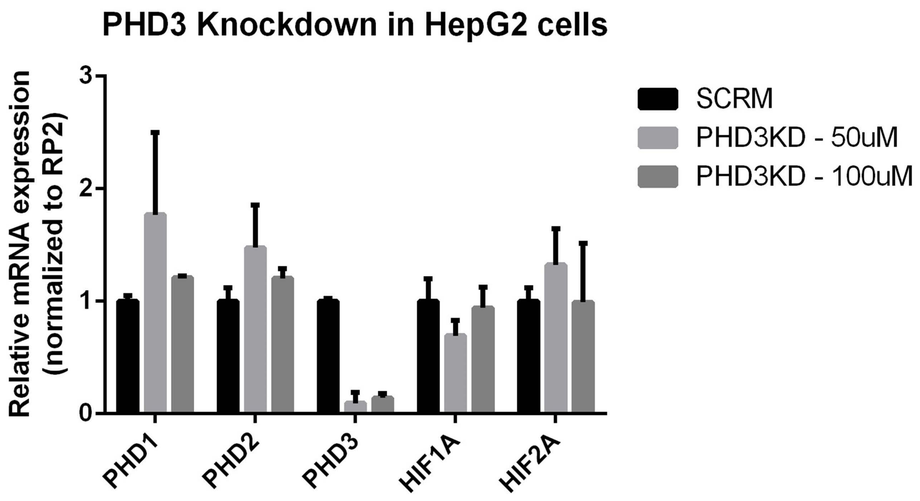

How much a gene is expressed can be measured by how much mRNA is present in a sample, since proteins are synthesized using mRNA. In qRT-PCR, mRNA is reverse transcribed to cDNA. However, samples of DNA are too small for machines to measure accurately, which is why PCR is used to amplify a certain region of DNA in a sample and quantify how much that gene is being expressed. The core of the process is PCR.

PCR is used to amplify a region of DNA until there is enough to be measured accurately, by using enzymes' natural processes to amplify the DNA. A DNA sample is mixed with DNA polymerase, the enzyme responsible for DNA replication, and mixed with primers, which are DNA segments that contain small complementary sequence of the target DNA. The DNA, with the primer and polymerase, is repeatedly heated and cooled so that the reaction can take place.

When the DNA is heated, the DNA "melts" and the two strands separate. The temperature is then lowered and each strand is used as a template for a new strand of DNA to be replicated by the polymerase. Every time this is repeated, the amount of a strand of DNA increases exponentially. Primers make the amplification process specific by providing polymerase a "starting point" for replication, since polymerase can only add new nucleotides to an existing strand of DNA.

Real time PCR differs from PCR in that it uses non-specific fluorescent dyes (SYBR green) that intercalate as DNA replicates. The dye binds to double-stranded DNA in PCR, causing the dye to fluoresce. As the amount of DNA increases during PCR, the intensity of fluorescence increases. After each cycle, a detector measures the fluorescence until the PCR is complete. Afterwards, it gathers all of the data for analysis by the experimenter.

## Results




## Discussion

The goal of this part of the project was to knock down Phd3 gene expression. If the knockdown was successful, we should now see a marked decrease in Phd3 mRNA abundance and no change in the expression of Phd1 and Phd2.

As expected, the graph shows marked decrease in Phd3 for the knockdown cells and relatively even expression for the other Phd proteins. There is a little bit of variety, but since those genes were not targeted, it is possible due to natural variation or pipetting error.

However, this study still has a long way to go. qPCR was used to verify that Phd3 expression was knocked down, and this knockdown occurs at the level of mRNA. However, the goal of this project is to observe the effects of decreased Phd3 expression of HIF-2a in HepG2 cells. The effects of lower Phd3 presence may not influence HIF-2a expression at the level of mRNA, but will influence it at the protein level, so the qPCR results do not give us the full picture. Examination of protein expression in HepG2 cells using the Western blot technique will follow the results of the qPCR to see if the desired effects on HIF-2a protein are being achieved.

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