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Characterization of In Vitro Models and Testing of RNAi Treatment for Achondroplasia

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Abstract

Achondroplasia is a form of short-limbed dwarfism caused by a highly conserved, Gly-Arg mutation in the transmembrane region of fibroblast growth factor receptor 3 (FGFR3). [Rousseau et al, 1996] The goal was to infect different cell lines with lentivirus carrying shRNA to FGFR3, and quantify the knockdown of FGFR3 mRNA. To do this, establishment of a proper cell line was necessary. Mouse embryonic fibroblasts (MEFs) and C3H10T1/2 cells were differentiated for 12 days to determine maximal expression of FGFR3. The MEF cells were then infected with the lentiviral vector, and FGFR3 levels were again analyzed. Knockdown was observed, as expected, in the MEFs, with an overall decrease of 90% over uninfected. It thus appears that the shRNA, delivered by lentiviral vector, decreased the amount of FGFR3 being expressed. In the future, a more specific sequence against mutant FGFR3 can be engineered, and viral infection of this sequence could down regulate the expression of the mutFGFR3, while sparing the wild type FGFR3, reestablishing bone growth in achondroplastics.

Acknowledgements

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Introduction

Achondroplasia (ACH) is a disease afflicting approximately 1 in 15,000 live births, and accounts for more than 90% of all diseases resulting in dwarfism. [Rousseau et al., 1996, Waller et al., 2008] The disease is brought about by a disruption in the process of endochondral ossification in the growth plate. Endochondral ossification is the process by which long bones are formed. It begins when mesenchymal cells condense to form chondrocytes. These chondrocytes then undergo a process of further maturation and progress to hypertrophic chondrocytes, which are characterized by an increase in size, formation of a cartilaginous matrix, and secretion of molecules such as vascular endothelial growth factor (VEGF), which allows for the formation of blood vessels. The invasion of blood vessels allows for osteoclasts and osteoblasts to invade and remodel the cartilage matrix and form mineralized bone. [Cameron et al., 2009] The result for achondroplastics is that their bones don't grow properly at their growth plates. They experience rhizomelic dwarfism, where the hip and shoulders are truncated, exaggerated lumbar lordosis, stemming from the lack of growth in their vertebrae, and minimal proliferation of the growth plate cartilage of long bones. [Naski et al., 1996, Rousseau et al., 1996, Deng et al., 1996] Essentially, wherever the bone is supposed to become longer, achondroplastics experience little to no growth. This results in an extremely short stature as compared to individuals without the disease (Figure 1). Patients suffering from Achondroplasia are still, however, able to reproduce, and have normal intelligence, with a mean life span of 61 years, compared to a mean general population of 71 years of age. [Waller et al., 2008]

Figure 1: A picture of achondroplastics taken at the turn of the century. [Carr, 2005]

This common form of chondrodysplasia is now known to be caused by one of two point mutations (G1138A or G1138C) at chromosome locus 4p16.3. The mutation causes a Gly-Arg substitution at position 380, located in the hydrophobic domain of fibroblast growth factor receptor 3 (FGFR3). [Vajo et al., 2000, Rousseau et al., 1994]

FGFR3, like most fibroblast growth factor receptors, consists of 3 extracellular immunoglobulin-like domains, for binding of the signal peptide, usually acidic and basic fibroblast growth factors, a single transmembrane segment, for propagation of the signal through the membrane, and a cytoplasmic tyrosine kinase catalytic domain (Figure 2). [Bruford, 2009] As with other receptor tyrosine kinases, the ligand binding creates a dimerization of two FGFR3 enzymes. This allows the two catalytic domains of the cytoplasmic region of the receptors to add

Fig. 2: Schematic representation of fibroblast growth factor receptor 3. The protein consists of several immunoglobulin domains (Ig 1-3), a transmembrane region (TM), and a cytoplasmic catalytic domain made up of two kinase sites. The mutation causing Achondroplasia is a G380R substitution located in the transmembrane domain of the protein.

phosphates to each other, a process known as autophosphoralation. This serves two main purposes. Firstly, the adding of phosphates to the cytoplasmic tails increases the kinase activity of the enzyme. Secondly, and mainly, the phosphoralted tyrosines surrounding the catalytic sites of the cytoplasmic tails become excellent docking stations for intracellular proteins that in turn become activated, propagating the signal initiated by the binding of the ligand to the receptor. [Alberts, Johnson, Lewis, Raff, Roberts, and Walter, 2007] The signal that is propagated continues along a path of kinases and transcription factors, in an effort to control cell processes through the transcription of DNA to produce RNA, and the translation of the mRNA into proteins (Figure 3).

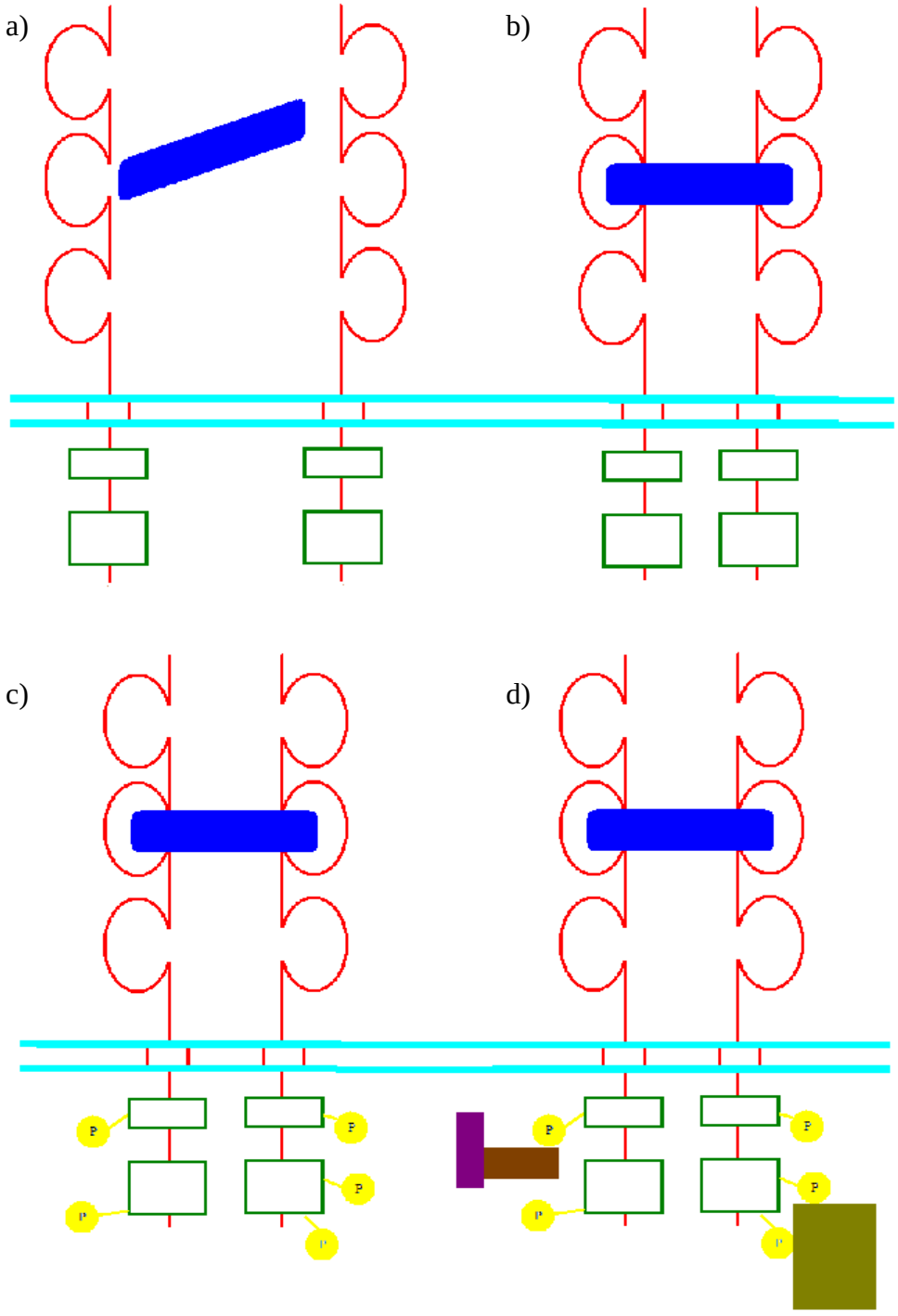


Figure 3: FGFR Tyrosine Kinase Autophosphoralation a) Two receptor tyrosine kinases (FGFRs) interacting with ligand. b) Dimerization. c) Dimerization activates autophosphoralation. d) Phosphates attract and activate proteins in the tyrosine kinase cascade pathway.

$FGFR_3$ has been found to regulate endochondral ossification through a negative mechanism, essentially limiting, rather than promoting, osteogenesis. In 1996, Deng et al. working at Harvard University used murine knockouts of the FGFR3 gene to discover that the knockout mice displayed characteristics opposite to the dwarfed phenotype expressed by human achondroplastics; the mice showed prolonged, accelerated bone growth and endochondral ossification. The scientists, therefore, suggested that the mutant variant of FGFR3 apparent in Achondorplasia does not create a loss-of-function in the FGFR3, but is a ligand-independent activation of the receptor. [Deng et al., 1996] As the ACH substitution is located in the transmembrane portion of the protein, it is theorized that the tyrosine kinase function is altered by the replacement of a hydrophobic guanine with a highly charged arginine. The receptor subsequently dimerizes as a result of the mutation without any fibroblast growth factor binding to initiate the dimerization. [Rousseau et al., 1994] Therefore, this highly conserved mutation (97% of all achondroplastics) creates a dominant negative mutant protein, designated $mutFGFR_3$. [Vajo et al., 2000, Naski et al., 1996] Because of the already negative mechanism of wild type FGFR3 (wtFGFR3), the mutant receptor enhances the limiting nature of the receptor, almost completely halting endochondral ossification in the long bones, and resulting in an achondroplastic's dwarfed stature. [Naski et al., 1996, Deng et al., 1996] One can, therefore, gather that if the mutFGFR3 was eliminated from the body, while sparing the wild type receptor, the result would show normal bone growth and endochondral ossification.

Stemming from this, our goal begins to become apparent. A lentivirus could be engineered with a small hairpin RNA (shRNA) vector, in order to activate RNA *interference* in cells expressing the mutant receptor. RNA *interference*, or RNAi, was first observed in 1986 when Rich

Jorgansen of the University of Arizona attempted to insert extra color purple genes into the genome of the petunia. [Fine, 2005] The resulting flower, however, showed no color whatsoever. After years of hypothesizing and investigating, scientists developed the solution; there must have been a mechanism in the cell that viewed the transcribed, extra purple gene products as foreign, and concordantly destroyed them.

The theory of RNAi was born. Pioneered by Craig Mello, using *Caenorhabditis elegans* and Philip Zamore, using *Drosophila melanogaster*, RNAi has since been catapulted into the spotlight. It was discovered that there are certain enzymes and proteins in the cell, Drosha, Dicer, and RISC, which seek to eliminate double stranded RNA (dsRNA) from host cells. Once dsRNA is found, Drosha, an RNAase, creates small hairpin RNAs (shRNAs) from the dsRNA, sometimes also referred to as pre-micro inhibiting RNA (pre-miRNA), by cleaving the strands at certain positions, and chewing the strands into 21 nucleotide fragments. Dicer, another RNAase, then splits the hairpins into two, complementary strands. The resulting single stranded RNA (ssRNA) fragments are then incorporated into the RNA-induced silencing complex (RISC), and used as a template for seeking out, not only foreign RNA complement to the sequences, but any RNA in the cell that resembles that sequence. It is for this reason that the petunias turned white; the extra purple mRNA made the cell attack any mRNA signaling the cell to make color purple proteins

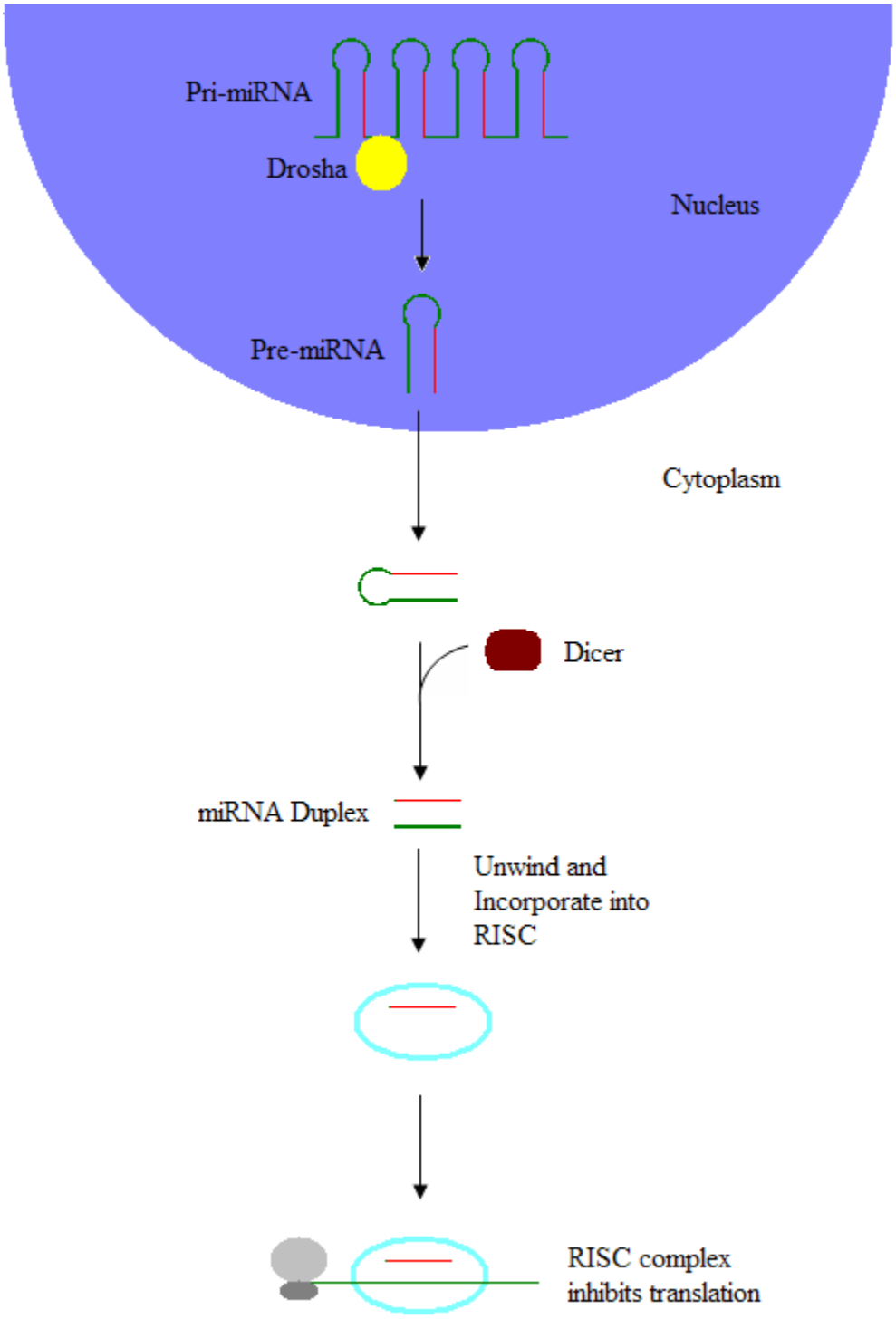


Figure 4: RNA interference pathway using hairpin miRNA. Drosha cuts the Pri-miRNA into Pre-miRNA, which is exported out of the nucleus. Then Dicer cleaves the hairpin, creating a miRNA duplex. When the complement to the target strand is incorporated into the RISC complex, translation is halted on the complement mRNA strand.

The real amazement, however, is that RNAi can be used as a way to silence gene expression. The mechanism is simple: engineer a small hairpin RNA (shRNA) containing a sequence complementary to the mRNA being targeted. Once introduced, the hairpin will be "seen" by the host cell as foreign, and will be cleaved, cut, and chewed by Dicer. The resulting fragments will then be incorporated into the RISC complex, and, subsequently, will activate RNAi, destroying any mRNA complementary to the chewed sequences. [Mello et al, 2006]

In 2006, Andrew Fire and Craig Mello won the Nobel Prize in Medicine for discovering this fundamental mechanism for controlling gene expression using RNAi. In Mello's lecture upon receiving the award, he stated that he "encourages [us] all to think about the possibilities, to learn more about biology and RNAi, and if [we] get inspired and excited, please join the adventure and help explore the many unknowns that are still waiting to be addressed." [C. C. Mello, public presentation, December 8, 2006] It is this calling that brings us to the project at hand: treating Achondroplasia using RNAi. Because the mutation (G1138A or G1138C) is so highly conserved within the population of achondroplastics, injecting a hairpin RNA sequence complementary to the mutant fgfr3 mRNA sequence will activate RNAi against the mutfgfr3 mRNA, ensuring deletion of mutFGFR3, while sparing wtfgfr3 mRNA. This, therefore, would reestablish the growth plates back to a state of normal growth, because only the wtfgfr3 mRNA would be available for translation.

Materials and Methods

Mouse Embryonic Fibroblasts were obtained from day E12.5 C57/Bl6 wild type mice. C3H10T1/s cells and MEFs were cultured in Dubelcco's Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). For differentiation, cells were plated in high-density micromass cultures at a concentration of 10^7 cells/ml. Micromass cultures were maintained in DMEM/F12 media supplemented with 5% FBS and 50nM BMP2. Then the cells were maintained in culture for 12 days and media was changed on days 2, 5, 8, and 11.

Cells were harvested on days 1,3,6,9, and 12 and total RNA was collected in Trizol. RNA was reverse transcribed into cDNA and FGFR3 mRNA levels were measured in the mouse embryonic fibroblasts (MEFs) and C3H10T1/2 (10T1/2) cells using quantitative polymerase chains reactions (qPCR), with cyclooxygenase (Cox) as our control mRNA, and SYBR Green I as the indicator. Visual analysis was then performed on the resulting data.

In order to add our shRNA to the MEFs, recombinant viral infections were done, and the subsequent knockdown was again measured by qPCR. The lentivirus containing shRNA sequences to FGFR3 had been prepared with a pGIPz (Open Biosystems) backbone, pLP1 (Invitrogen) packaging plasmid, and pVSV-G (Invitrogen) envelope plasmid. The cells were seeded on 100 mm in normal culture media, and left to grow for 24 hours. After 24 hours, cells were infected with lentivirus. The media was changed the next day and cells were visualized under and Immunoflourescense microscope to determine GFP expression and infection efficiency. Cells were then plated in high-density micromass culture as described above. Cells

were harvested on day 5 and total RNA was isolated in Trizol. RNA was reverse transcribed into cDNA and FGFR3 mRNA levels were determined by qPCR.

Results

In order to attempt to treat Achondroplasia in humans, much testing must be conducted in vitro and in vivo, in various cell and animal models, and the process must be shown to be successful in these models before human testing can be attempted. The first task, therefore, was to find a proper cell line in which to conduct the lentiviral infections of the shRNAs. We looked into various cell lines that we believed were similar to the chondrocytes affected in Achondroplasia. C3H10T1/2 cells are mesenchymal progenitors which have the ability to differentiate into a variety of different bone precursor cells, such as osteoblasts and chondroblasts. Derived from early mouse embryos, C3H10T1/2 cells have been used extensively as in vitro models to examine mesenchymal differentiation into various phenotypic lineages by different inductive mediators. [Shea et al., 2003] Our primary cell line, however, was the mouse embryonic fibroblast line (MEFs). MEFs had been used for many years to study growth control and DNA damage. However, in 2004, the Department of Cell Biology at the University of Massachusetts Medical School demonstrated MEFs in virto can enter, and complete, chondrogenic differentiation, or chondrocyte maturation. They showed that the cells could be induced by bone maturation proteins to form 3-dimensional (3-D) cartilaginous tissue. Their research established the MEF as a powerful tool for the study of gene function during chondrogenesis. [Lengner et al, 2004]

In order to measure fgfr3 mRNA expression levels accurately, qPCR was conducted on prepared MEF and C3H10T1/2 cell aliquots. To achieve the greatest amount of expression, we differentiated the cells for 12 days in BMP-2, in the hopes that the later stages of endochondral

ossification would show higher levels of fgfr3 mRNA expression. After having differentiated the cells for 12 days, qPCR was run to show the relative expression levels of the cells at different time points (Day 0, Day 1, Day 3, Day 6, Day 9, and Day 12). The results were then analyzed, and are displayed in Table 1 and Figure 5. The expression levels can be seen to increase as time progresses. In other words, at later differentiation points, there was a greater expression of fgfr3 mRNA. In the C3H10T1/2 cells, the maximal expression level occurred at Day 3, with a relative value of 139.74, compared to Day 0 expression levels in that cell line. However, in the MEFs, maximal expression was seen at Day 9, with a much greater value of 1307.87 when compared to Day 0 expression levels of the C3H10T1/2. Therefore, we inferred that the MEF cells at Day 9 showed the greatest expression of fgfr3 mRNA.

Sample	**Detector**	**Relative**	**Diff (+)**	**Diff (-)**
10T1/2 d0	mFGFR3	1.00	0.29	0.22
10T1/2 d1	mFGFR3	5.05	2.92	1.85
10T1/2 d3	mFGFR3	139.74	31.11	25.44
10T1/2 d6	mFGFR3	58.83	26.77	18.40
10T1/2 d9	mFGFR3	61.91	18.17	14.05
10T1/2 d12	mFGFR3	41.80	12.60	9.68
MEF d0	mFGFR3	1.33	0.32	0.25
MEF d1	mFGFR3	3.41	1.10	0.83
MEF d3	mFGFR3	12.86	15.09	6.94
MEF d6	mFGFR3	299.30	109.65	80.25
MEF d9	mFGFR3	1307.87	726.17	466.92
MEF d12	mFGFR3	353.63	164.84	112.43

Table 1: qPCR Analysis. The table shows relative expression levels of fgfr3 mRNA.

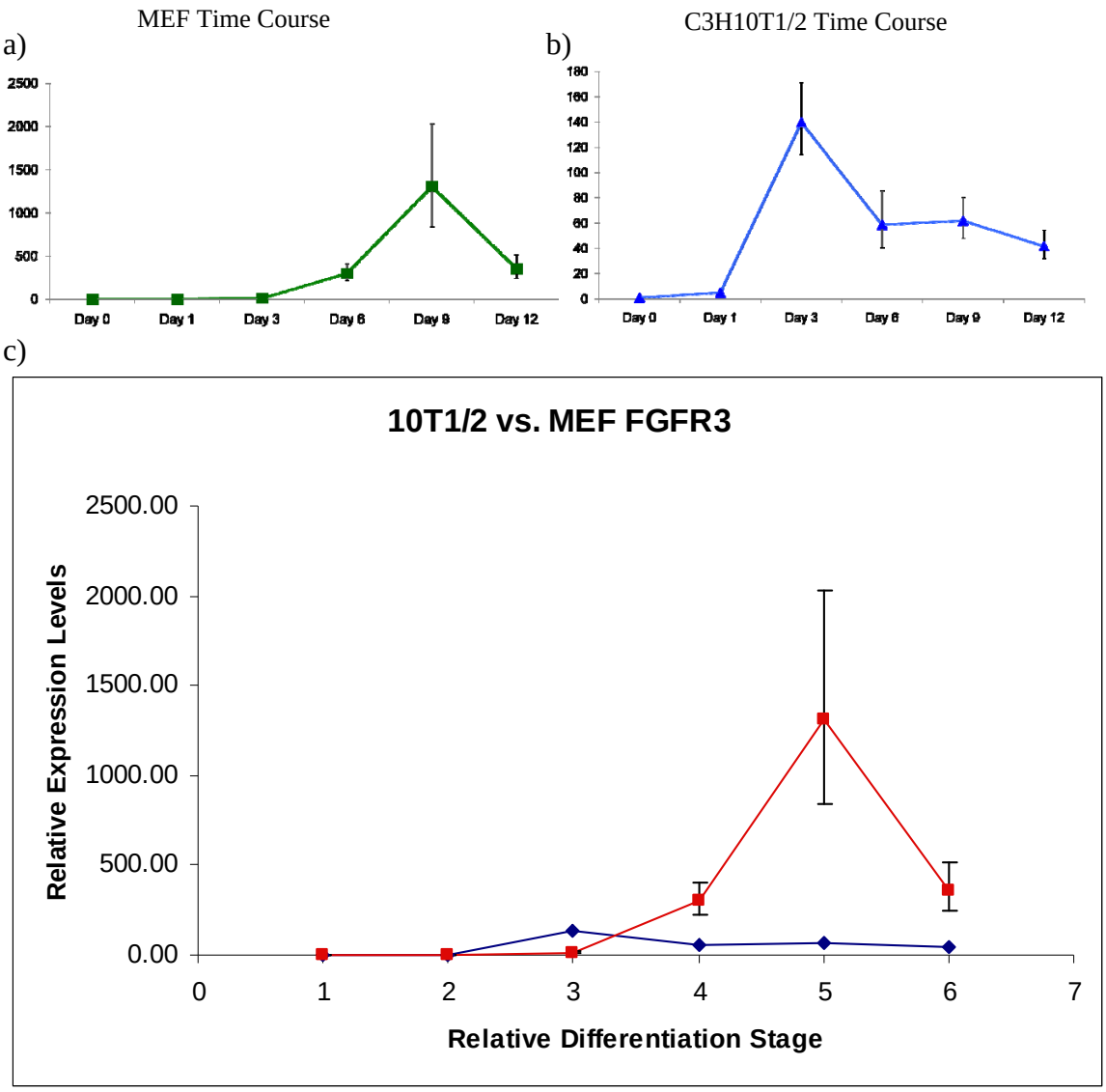


Figure 5: a) Profiling of FGFR3 RNA levels through chondrogenic differentiation of MEFs b) Profiling of FGFR3 RNA levels through chondrogenic differentiation of C3H10T1/2 c)Relative Expression Levels of MEF (red) to C3H10T1/2 cells (blue). Highest expression level was seen in the MEFs at differentiation stage 5 (day 9).

To demonstrate that knockdown could be shown using an RNAi pathway, fgfr3 mRNA levels were measured before and after infection of our lentivirus/shRNA vector, using a non-silencing (NS) lentivirus as a control. Although the process showed knockdown from our NS virus, further knockdown was observed in cells infected with lentivirus carrying our shRNA vector. Therefore, it can be supported that the shRNA vector activated RNAi, and knocked down fgfr3 mRNA transcription.

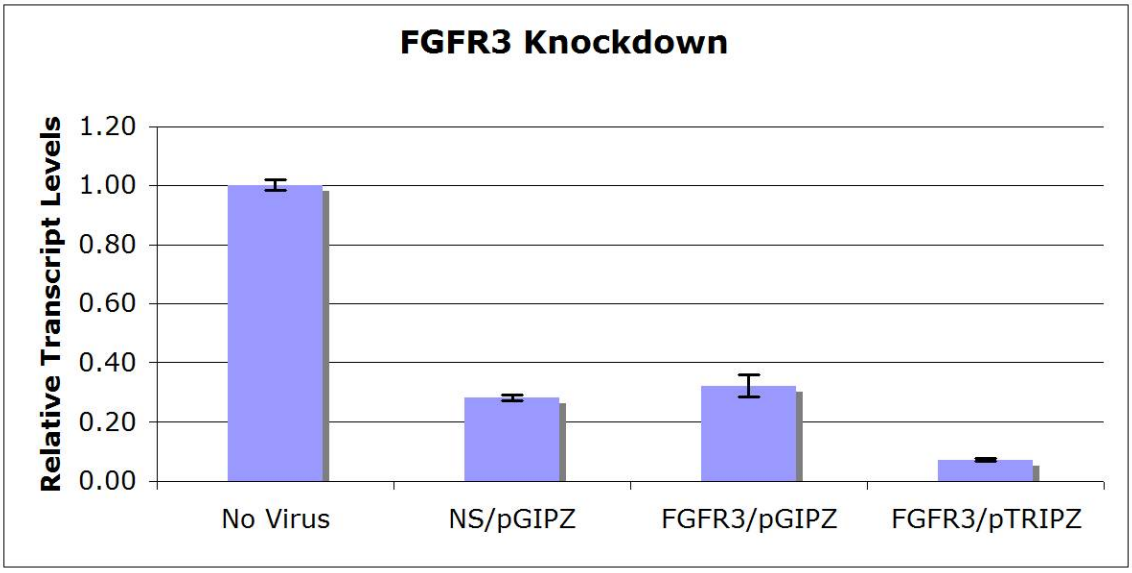
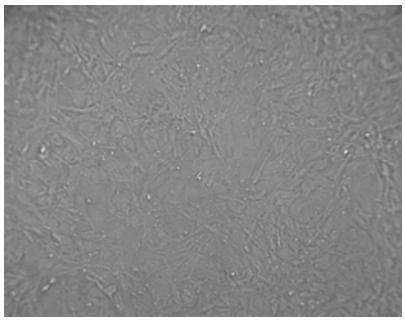
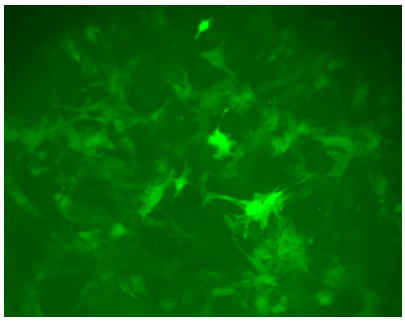


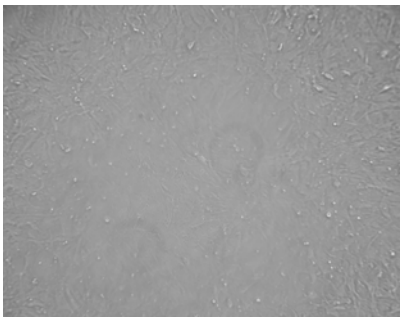
Figure 6: Fgfr3 Knockdown levels in MEFs using relative transcription levels. Although the non-silencing lentivirus (NS/pGIPZ) knocked down mRNA expression in the MEFs, a 3 fold decrease is still observed between the NS and the pTRIPZ knockdown in which our vector was transfected.

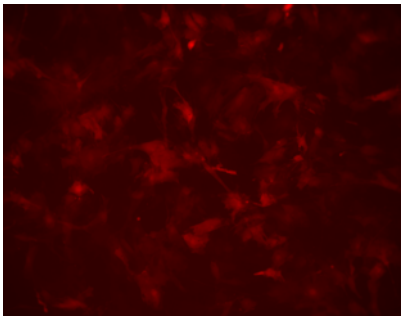
FGFR3/pGIPZ



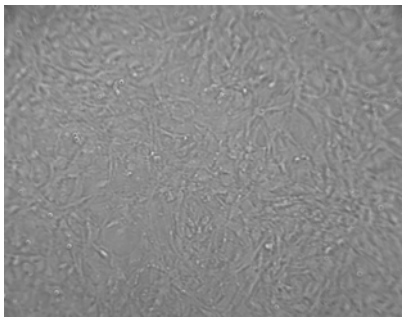


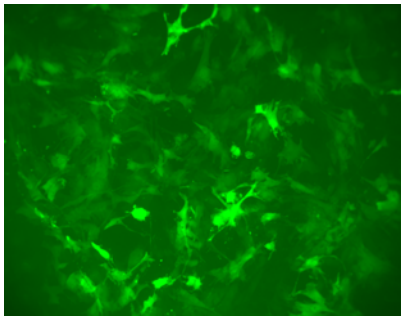
FGFR3/pTRIPZ





NS/pGIPZ





Discussion

The implications brought forth by this study are great in the advancement of a treatment for Achondroplasia. In successfully showing that chondrocyte cells in vitro can be manipulated to differentiate and express fgfr3 at maximal levels (Figure 5) gives us a great advantage in observing whether fgfr3 is knocked down significantly. In infecting the cell line, and successfully knocking down fgfr3 mRNA expression levels, we are one step closer to treating Achondroplasia in humans.

However, one thing that must be overcome is the non-silencing (NS) knockdown that was evident in the lentiviral infections of the MEFs (Figure 6). The process used to plate the MEFs for infection, micromass, is highly temperamental. However, we hypothesize that in infecting the cells so early in the growing process, the cells became unstable, and the knockdown observed by the NS lentivirus, was a systemic knockdown of all mRNA transcription. To overcome this, we will attempt to let the cells become adapted to the plate and media, and, at a later point, infect with the lentivirus. This will not only serve to show whether we can eliminate such a great NS knockdown, but also incorporate the research of the differentiation stages of the cell lines into the knockdown equation. With MEFs, infection at Day 7 would be most probable, as it was at Day 9 that maximal expression levels were observed. Hopefully, the cells will be adapted enough to the process that transcription will not be affected by the non-silencing lentivirus, and only affected by lentivirus carrying our shRNA vector. This will then, hopefully, show that our shRNA vector activates RNAi in the cell, and show subsequent knockdown of fgfr3 expression.

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