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Silencing of a novel candidate gene involved in bone metabolism, in vitro

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Title (English)

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Title (Swedish)

Abstract

Osteoporosis is a disease with growing indices in the society of today. To a large extent it is genetically regulated and several candidate genes have been proposed to be involved in the establishment of inter-individual variation in the process of bone metabolism. *Wnt Inhibitory Factor 1 (WIF1)* is known to regulate a group of molecules, Wnts that have been identified as important for bone formation. This degree project is part of the mapping of *WIF1* and involves the silencing of the gene, *in vitro* in bone cell culture. Gene-specific siRNAs were introduced by Magnet Assisted Transfection and gene expression was subsequently evaluated by real-time quantitative PCR. Results revealed that the target gene was successfully silenced with up to 98 % silencing measured, suggesting possibilities for further characterizing the function of this gene and how it is involved in the mechanisms of bone metabolism.

Keywords

Osteoporosis, RNAi, Wnt-signalling, WIF1, Bone cell culture, MATra

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Sammanfattning

Osteoporos, även kallat benskörhet har kommit att bli en av våra vanligaste folksjukdomar av idag. Upp till hälften av alla kvinnor och en tredjedel av alla män drabbas någon gång under sin livstid av en fraktur till följd av osteoporos. Flertalet olika faktorer bidrar till utvecklingen av osteoporos hos en individ vilka framförallt är ålder, medicinering och kvinnors menopaus. Utöver dessa finns dock flera genetiska faktorer. Idag vet man att våra gener till stor del påverkar benets fenotyp (egenskaper) och att olika individer därför har en unik benfentyp. Inom ben-forskningen har man till och med kunnat koppla specifika geners uttryck till osteoporotiska benfenotyper.

Detta arbete är en del av forskningen inom ben-genetik och innefattar tystandet av en ben-specifik gen i cellkultur. Tystningsförsöken av genen utfördes på humana benceller uppodlade ur benvävnad från patienter på Akademiska sjukhuset i Uppsala. Målgenen tystades framgångsrikt med siRNA och en ny metod för introduktion av dessa i cellerna, ”Magnet Assisted Transfection”. Det intressanta är att om man kan tysta en gen på detta sätt är det även möjligt att bestämma genens funktion i relation till andra gener i vårt genom. Resultatet från denna studie gör denna typ av försök möjliga att utföra i framtiden.

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Contents

Abbreviations

BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
cDNA	complementary Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxid
FCS	Foetal Calf Serum
FMCA	Fluorometric Cytotoxicity Assay
MATra	Magnet Assisted Transfection
miRNA	micro RNA
qPCR	Quantitative Polymerase Chain Reaction
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RNAi	RNA Interference
SFRPs	Secreted Frizzled-Related Proteins
siRNA	Small Interfering RNA
WIF1	Wnt Inhibitory Factor – 1
Wnt	Wnt protein

1. Introduction

Understanding the role and function of genes is crucial for a number of reasons. In the field of medicine, this could translate into finding information on mechanisms causing disease. Since molecular mechanisms in cells and organisms are complex, it is necessary to study as many aspects of gene function as is possible. A common way of determining gene function is association studies between genotypes and phenotypes. It is well established that distinct genotypes can give rise to different phenotypes, making genotypic association studies a powerful tool within the genomics research field. Commonly, animal model systems are used to find genetic effects on phenotypes of interest, and from there research is translated into human systems.

Since bone diseases are common in our society and there are many genetic factors known to have an influence on bone metabolism, research is central within the field. The following project is part of the research within the bone genetics group by the Department of Medical Sciences at Uppsala University Hospital, where research is focused on the genetic impact on bone diseases. Within the group, novel candidate genes for bone phenotypes, such as bone mineral density and bone biomechanical strength phenotypes, have been found by inter-cross studies between two chicken strains, wild type and domestic chicken. One of these candidate genes, *WIF1*, which is the gene of interest in this study, has a known human orthologue and is hypothesised to play a role in bone metabolism regulation, and probably also in other un-investigated molecular processes.

This master's degree project includes a series of experiments to optimize a technique of silencing the *WIF1* gene in human primary bone cell cultures and MG63 cell cultures *in vitro*. The actual silencing effect was achieved by RNA interference (RNAi) and the transfection method used was Magnet Assisted Transfection (MATra).

2. Background

2.1 Osteoporosis

Osteoporosis (brittleness of the bones) has over the years become one of our most common diseases and is known to afflict up to as many as 50% of all women and up to a third of all men during their life time (1). The main reason for this is the fact that our population is getting older and older. Osteoporosis is accompanied by an increased susceptibility to bone fractures, which is due to reduced bone mineral density (BMD) in the skeleton and degradation of bone tissue. In practice, there are two different types of osteoporosis, one that is called postmenopausal osteoporosis (Type I), which affects women and is due to the natural hormonal changes that occur in connection with the menopause. Senile osteoporosis (Type II) afflicts both men and women and is usually caused by ageing in combination with other various factors. Medication with cortisone or insufficient uptake of calcium and D-vitamin, and smoking are some of them, accelerating the course of bone loss. Besides this, daily life, to a large extent, can affect the susceptibility of brittleness of the bones. For example, it is known that bone mass increases up to 25 years of age, and thereafter it is reduced. During this first period of life it is important to build a strong skeleton by exercising (putting strain on the skeleton increases the bone formation), eating right, to have a sufficient calcium uptake, and avoid for example smoking. The outcome of osteoporosis is fractures of bones, but if not prevented or if left untreated, it can progress painlessly until the time of fracture. Typical fractures for Type I osteoporosis are on the spine and wrist, while fractures most common when suffering from Type II are in the hip, the proximal humerus, the tibia and the pelvis in elderly women. Type II is due to a proportionate loss of both trabecular* and cortical* bone (2,3,4).

* Cortical bone is dense and forms the surface of the skeleton and thus contributes to 80% of bone mass. In contrast, trabecular bone has low density and strength but instead a very high surface area that fills the inner cavity of long bones. Trabecular bone is also called spongy bone.

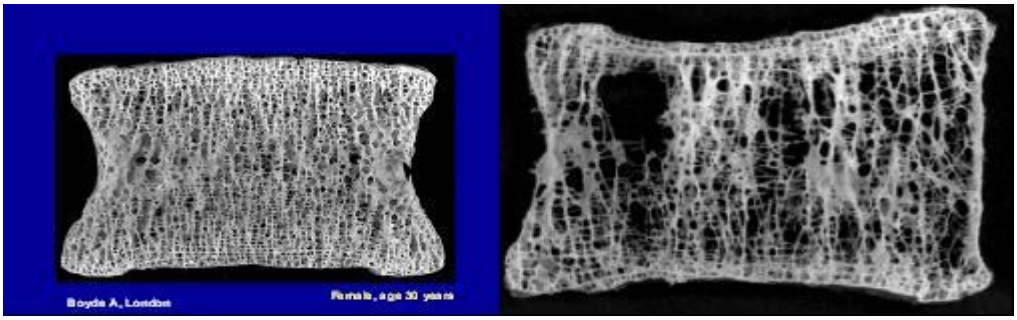

Figure 1: Bone of a female, 30 years old (to the left) and bone of a 70 year old female (to the right) visualizing the natural process of bone loss when ageing (Permission granted by Andreas Kindmark, Uppsala University Hospital).

Even though environmental factors are obvious for osteoporosis, the importance of genetic factors should not be belittled. As much as 70%-80% of the variance of bone mineral density is explained by genetic factors (5,6,7). Different bone phenotypes have been associated to various genotypes and the genetic influence on osteoporosis has been shown in several studies, both when it comes to poor bone structure and the ability to sustain fractures. It is proposed that the importance of genetic factors depends on where the fracture is located and the age of the patients. Thus, the search for susceptibility genes and environmental factors, that may modulate expression of these genes, in younger elderly patients with hip fracture, the most devastating osteoporotic fracture, should be encouraged (8).

2.2 Bone

Bone tissue is a mineralized connective tissue containing partly an organic phase called the matrix and an inorganic, crystal-like phase (7,9). Mainly, there are three different cell types represented in bone; osteoblasts are the cells responsible for building bone while osteoclasts resorb bone and osteocytes are embedded in formed, compact bone, and are the most numerous of bone cells represented (7,9,10). During adult life, bone is constantly being remodelled and there is always a balance between mineralized matrix deposition by osteoblasts and matrix resorption by osteoclasts. This balance is crucial for the maintenance of a steady bone mass. Osteoblasts differentiate from pleuripotent mesenchymal stem cells and there are multiple factors contributing to this development. During the process several known genetic bone markers are expressed, e.g. alkaline phosphatase (ALP), osteocalcin, osteopontin and collagen. Genetic control of these markers is rigid and their expression pattern during the different developmental stages of osteoblasts is well established. A normal cell cycle of an osteoblast is three months as a bone builder cell and thereafter it naturally dies through apoptosis. Beyond these markers there are other interesting factors in the context of bone cell formation. Bone Morphogenetic Protein-2 (BMP-2) and factors of the Wnt system are known as

regulators of osteoblast development and these are useful in the studying of osteoblast formation and differentiation (7,9,10,11).

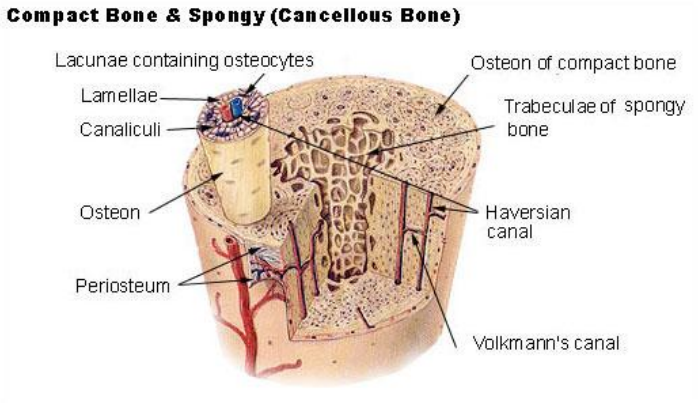

Figure 2: Cross section of bone where cortical bone (compact bone) is the outer, more dense type of bone and where trabecular is found in the inner part of the bone as a spongy type of bone. (www.wikipedia.org/wiki/cortical_bone)

BMPs are members of the transforming growth factor β (TGF β) superfamily of polypeptides, which include TGF betas, activins, and inhibins. The proteins display specific conservation between species and are synthesized by skeletal and extraskeletal tissues. BMP-2 is synthesized by osteoblasts and plays an autocrine role in osteoblastic function. They are unique and induce the differentiation of cells of the osteoblastic lineage. The signalling is induced by the interaction between BMP and two distinct serine/threonine kinase receptors. After this initiation, BMPs, TGFbetas, and activin signal through Smads. Further, Smads interact with Runx2/Cbfa-1 to establish effects on specific cellular components. Osteoprogenitor cells can form myoblasts, adipocytes or osteoblasts. Among the known stimulation factors that induce differentiation into osteoblasts are BMPs (7,12).

2.3 Wnt signalling

Wnt proteins belong to a family of secreted glycoproteins and regulate diverse biological processes including cell fate determination and polarity, cell proliferation and apoptosis. Recent data show that Wnts regulate bone cell fate choice, stimulate osteoblastogenesis and inhibit adipogenesis (the formation of adipocytes) from mesenchymal precursors in vitro and in mice. Signals are mediated by the binding to receptors in the Frizzled (Fzd) family and the low-density lipoprotein receptor-related proteins Lrp5 and Lrp6. So far, three Wnt signalling pathways have been identified; the Wnt/β-catenin (canonical pathway), the Wnt/planar cell polarity, and the Wnt/calcium pathway. The canonical pathway is related to osteoblast formation and is the best described, leading to the stabilization of β-

catenin. The initiation of intracellular accumulation of β-catenin by binding to cell surface receptor complexes consisting of Lrp5/6 and Fzd transmembrane proteins seems to be the basis for the Wnt protein's proliferation and differentiation-dependent effects. It is also understood that this mechanism inhibits glycogen-synthase 3 and thereby prevents degradation of β-catenin. In contrary, in the absence of Wnts, the β-catenin levels are kept at a steady state. β-catenin is an intracellular molecule that plays several important roles in skeletal development. Via its interaction with E-cadherin and α-catenin, it directly links cadherins and the cytoskeleton, and is therefore essential for both cell adhesion and migration. The role of β-catenin in the Wnt signalling pathway is described by seminal work, performed by several laboratories, showing that β-catenin is also the molecular node of the canonical Wnt signalling pathway. The cytoplasmic stabilization and accumulation of β-catenin, via canonical Wnt signalling, leads to β-catenin entering the nucleus and heterodimerizing with Lef/Tcf transcrpiption factors to regulate Wnt target genes (4,13,14,15).

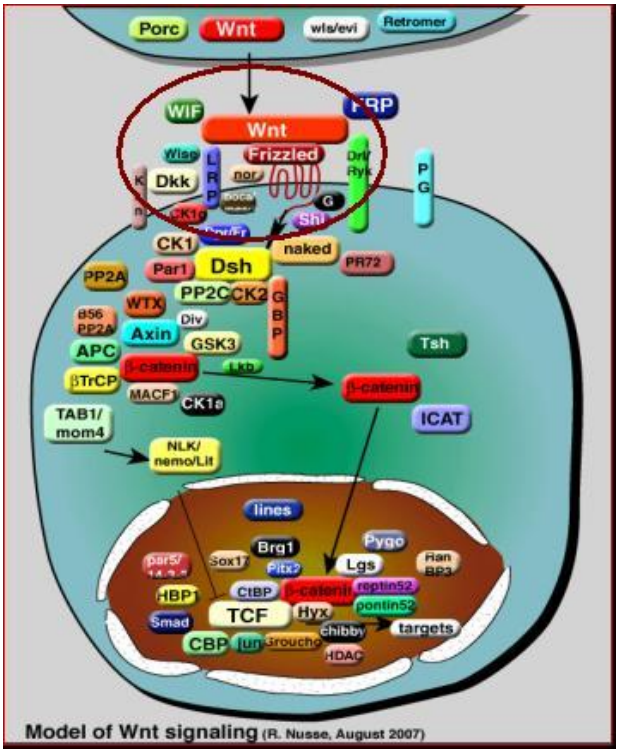

Figure 3: The Wnt signalling system, canonical pathway. Wnt proteins are secreted proteins that bind to membrane-receptor proteins and activate the Wnt signalling cascade, stabilizing β-Catenin in the end. (Permission granted by Roel Nusse, Stanford University, California).

Disruption of Wnt signal-transducing proteins is known to cause inherited disorders in humans. For example familial adenomatous polyposis coli (*APC*), a condition where polyps are formed in the colon, familial exudative vitreoretinopathy (FAV), caused by mutations in the receptors *FZD4* and *LRP5*, which is a rare eye disease affecting the retina and the clear fluid inside the eye. There is tetra-

amelia, caused by a mutation in *Wnt3*, causing malformations and even lack of arm and leg bones. Further, it is documented that loss of function in the Wnt-receptor Lrp5 leads to the severe osteoporosis pseudoglioma syndrome (OPPG) but also that a specific point mutation in *Lrp5* results in extremely high bone mass (HBM). Interestingly in the latter defect, bone is fragile, and not rigid, in spite of the fact that the bone is dense (16, 17, 18).

2.4 *WIF1*

Aside from mutations in components of the Wnt system, there are molecular effects causing disturbances of Wnt signalling. In 1999 there were two protein families known to inhibit Wnt proteins in their action; the secreted Frizzled-related proteins (SFRPs) family and the Dickkopf (Dkk) protein family. The SFRPs belong to a class of Wnt antagonists and SFRP-1 is highly expressed during the transition from pre-osteoblasts to osteoblasts. Nowadays, Wnt Inhibitory Factor 1 (*WIF1*) is also one of the proteins known as an antagonist to Wnts. Both WIF1 and SFRPs bind directly to Wnt proteins thereby preventing receptor activation. The history about *WIF1* tells us that it was first identified as an expressed sequence tag from the human retina. Thereafter, conserved orthologues were found in mouse, Xenopus and Zebrafish (19).

Wnt Inhibitory Factor 1 (*WIF1*) has been reported a few times in the context of bone research and is thought to play an important role in Wnt-signalling e.g. in the canonical pathway. The protein is secreted (20) and thereby a circulating factor known to inhibit Wnt-protein activity by blocking the ability to bind their receptors in the Wnt-signalling pathway, affecting the outcome of β-catenin activity.

Recent data from research on Wnt signalling show that Wnt signalling genes are differentially expressed between non-differentiating and differentiating osteoblasts (24). It has been demonstrated that Wnt signalling stimulates early steps of osteoblast differentiation and inhibits more mature osteoblasts. These are conflicting data but were demonstrated within the same osteoblast differentiation model. It was found found that differentiating osteoblasts have suppressed Wnt signalling and changed expression of various Wnt signalling genes, which might help facilitate the differentiation and mineralization process in osteoblasts. The authors have included Wnt inhibitory factor 1 (*WIF1)* among the genes analysed for expression on Affymetrix Gene Chips. The gene did not show any significant up or down regulation either in differentiated or non-differentiated cells, despite

the earlier reported detection of *WIF1* among osteoblast markers by microarray technology, and the fact that *WIF1* was found to be expressed during late-phase osteoblast differentiation (21,24,25).

Hence, *WIF1* has been shown to be expressed during a late stage of osteoblast differentiation in vitro but also in osteoblasts during in vivo bone development. The latter was shown by in situ expression analysis in newborn murine tail vertebrae. Another bone density study made on mice showed that the lowest bone mineral density was present in mice with the highest *WIF1* transgene expression levels. The gene expression data for W*IF1* in mice and humans, together with the low BMD phenotype of *WIF1* transgenics, suggests that *WIF1* plays a role in bone biology as a negative regulator of bone mass (21). Studies show that *WIF1* expression is high in trabecular bone but not in cortical bone, which is interesting since trabecular bone is renewed more often because of its considerably faster turnover rate than cortical bone. Furthermore, *WIF1* was found to be differentially expressed in females of two different chicken strains, White Leghorn (WL) and Red Junglefowl (RJ), where WL comprised higher *WIF1* transcript levels than RJ and also exhibited higher BMD (22). Additionally it has been shown that overexpression of *WIF1* decreases bone density, leading to an increased susceptibility to bone fractures (23). Together, these findings imply the importance of extended research about *WIF1* and its role in bone metabolism. It also suggests that the genetic variance could play an essential role for which bone phenotype an individual resides and what susceptibility they will have for osteoporosis.

2.5 RNAi

RNA interference (RNAi) is the mechanism where small RNA molecules target complementary RNA sequences in the transcriptome and launch the degradation of mRNA, inflicting decreased gene expression of certain genes. The phenomenon was first noticed in plants through the use of inverted-repeat transgenic viruses (26) and thereafter it has been observed in all eukaryotes and also shown in different model systems in which gene expression was specifically silenced (27). MicroRNA (miRNA) molecules are important gene regulators in the endogenous RNAi process that naturally occurs in the cell, whereas small interfering RNA (siRNA) have diverse biological roles in antiviral defence or transposon silencing (28). Thus, the natural function of these small RNA molecules is thought to be an ancient protection for the organism against viral components, bacteria and other unknown particles entering the cell. The molecular mechanism of RNAi is depicted in Figure 5. Double stranded RNA (dsRNA) precursor molecules enter the cell and are recognized by a ribonuclease (RNase) Dicer, which has the function to degrade the dsRNAs into shorter, 21-23bp siRNA molecules. Specific proteins, e.g. the RNA-induced silencing complex (RISC) is then associated with the siRNA and

guided to the target mRNA, where enzymes in the protein complex degrade the mRNA sequence (27,28).

2.5.1 RNAi In Practice

RNAi has become a useful application in reverse genetics, the methodology in which one seeks to find possible phenotypes that may derive from a specific genetic sequence. In studies of gene expression and function, RNAi is revolutionary and for clinical research especially, it is now an interesting tool. The commencing efforts to use RNAi in mammalian cells proved a non-specific, interferon-mediated response to dsRNA longer than 30bp. After solving this problem by successful introduction of shorter RNA moleculeas, 21-22 nt long, RNAi was induced in mammalian systems without inducing the interferon response. The siRNAs were chemically synthesized or expressed from plasmid vectors (27). In practice, there are many examples of viral vectors, useful for the introduction of siRNA for this purpose, i.e. lentivirus and adenovirus. Other techniques such as electroporation, lipofectamin and magnet assisted transfection have also been used in the laboratories. The choice of technique to introduce siRNAs into target cells *in vitro* depends on cell type but also on the actual target gene sequence and the properties of the siRNA. Further, these systems have different abilities to retain the effect of siRNA. In general, viral vectors are considered as a long-term expression system in comparison to electroporation, which is a short-term expression system (28). However since little is known about the persistency of silencing by siRNAs, validation of effects is crucial in every experiment and there is no obvious choice of method.

Introduction of RNAi in mammalian systems requires the direct use of siRNA rather than dsRNA since dsRNAs provokes a potent viral response (27). The siRNAs are loaded into the RISC directly and guides the complex to the target mRNA. siRNAs are provided by different companies either pre-designed for a certain gene or after ordering a specific sequence. These siRNAs usually are 21 bp long with 3′ dinucleotide overhangs. A good experimental design is important when working with siRNA and is obtained by using several, at least two functional siRNAs independently targeting the same gene. This assures that the biological effect is due to specific silencing of the transcript and not offtarget effects. Studies on which properties of siRNAs are favourable have been performed recently. The authors used a technique of tailing a whole set of siRNAs against a target transcript to evaluate which siRNAs were the most efficient (3). The study ended up in a number of guidelines how to design functional siRNAs. These are listed as follows: There should be an A or U nucleotide in the 5′ end of the AS (anti-sense strand), a G or C nucleotide in the 5′ end of the SS (sense-strand). Further, it should be an AU-rich sequence in the 5′ terminal, a 7 bp long region of AS, and no GC repeats longer

than 9 bp length (29). The effect of targeting siRNAs is usually compared to negative controls where one introduces short RNA molecules that do not target the mRNA of interest. Commercial negative control siRNAs are thus non-targeting scrambled siRNAs with properties as equal to the functional siRNA as possible. The number of GC-nucleotides is a determining factor in the choice of negative control siRNA. There are low-GC-, medium-GC-, and high-GC siRNAs to fit the characteristics of the targeting siRNA.

For future perspectives, the challenge as it comes to RNAi in clinical research is how to introduce siRNA into target cells in vivo. There is no such obvious tool today how to direct siRNAs to a specific tissue. However, recent reports point out several possibilities and even examples when introducing siRNAs into a certain tissue has been successful (30). Both lipid- and polymer-based vehicles for systemic delivery of siRNAs have been developed and tested, and there are companies today having achieved stable delivery of siRNAs associated with such particles to the liver, pancreas, kidneys and some types of tumours (30). No method is yet perfect but the prospects within the field are positive and major research efforts will probably be carried out within the following decades.

3. Materials and Methods

Throughout the siRNA experiments two types of cells have been cultured, primary cells from human bone tissue (Hob-cells) and the commercial cell line MG63 (derived from human osteosarcoma). A pilot study where *WIF1* transcript levels were detected in untreated cells was performed in order to assess the detectability of the transcript levels and thereby being able to continue with siRNA experiments on the cells. Parameters for the optimization of the silencing effect were determined through several experiments. First, a titration experiment to determine the optimal siRNA dose for the desired effect was carried out. Second, a time course experiment to verify the persistence of the knock-down effect was performed. Additionally, experiments with untreated cells were carried out to verify if differing transcript levels were due to how many days the cells had been in culture or the actual cell numbers. MATra, the new highly effective transfection method using magnetic force to deliver nucleic acids to cells was used to deliver siRNA and the actual transcript levels were evaluated by TaqMan®Gene Expression Assays.

3.1 Cell culture

3.1.1 Primary bone cell culture

Human trabecular bone tissue was obtained from the orthopaedic department at the University Hospital in Uppsala. Bone tissue was seeded in T75 flasks in Minimum Essential Eagle Medium with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 1% Penicillin G at 37°C, 5% CO_2. Medium was refreshed once every week, and after three weeks, the bone tissue had produced a confluent layer of migrated cells from the tissue, at the bottom of the flasks. Having reached confluency, cells were passed into two new flasks and bone tissue was removed. Medium was refreshed twice a week and cells passed when confluent. The passage of the cells was performed by trypsination. For a T75 flask, cells were washed with 7ml Phosphate Buffer Saline (PBS) and thereafter covered with 1ml Trypsin and incubated for 2-3 min in 37°C, 5% CO_2. The cell suspension was diluted with cell medium and seeded in new flasks. The number of Hob-cells in one confluent flask is sufficient to be split into two fresh flasks for further culturing.

3.1.2 Human MG63 cell culture

MG63 (batch 020614) cells were stored at -150°C. Cells were quickly thawed by adding cell culture medium, Minimum Essential Eagle Medium with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 1% Penicillin G, to the cryotube, gently pipetting up and down, and then transferring the cell suspension into a 10ml falcon tube with additional medium. Cells were centrifuged at 300 x g for five minutes to form a cell pellet and remove DMSO. The pellet was re-dissolved in fresh medium, put in a T25 flask and incubated at 37°C, 5% CO_2 until confluency. MG63 was trypsinated every other day and one confluent T75 flask MG63 was split into four to five fresh flasks.

3.2 siRNA

A set of three different, pre-designed *WIF1*-siRNA (Invitrogen, Carlsbad, CA) and three Universal Negative Control siRNA (Invitrogen, Carlsbad, CA) were utilized in the silencing experiments. Two of the negative controls had low GC content while the third had medium GC content, corresponding to the properties of the chosen *WIF1*-siRNAs.

3.3 MATra

The MATra technique is performed on a magnet plate suitable for standard format cell culturing flasks and plates. For transfection of adherent cells, the MATra-A protocol is used, which provides the MATra-A reagent consisting of magnet beads in solution. The Promokine protocol MATra-A for adherent cells for transfection was used for the experiments And the reagent kit was supplied by IBA BioTAGnology, Goettingen, Germany. Cells were plated the day prior to transfection in 10% FCS medium. At the time of transfection, the nucleic acid was diluted in serum- and supplement-free medium and thereafter the Matra-A reagent was added, mixed thoroughly and incubated for 20 min at room temperature. During the incubation a medium change to serum- and supplement free medium was carried out in the plate wells, since transfection in serum free medium can enhance the uptake of nucleic acids into the cells. After adding the nucleic acid mixture into the wells and mixed well, the cell plate was incubated on the magnet plate for 15 min. The plate was removed from the magnet, the medium in the wells was exchanged to 10% FCS medium and the plates were incubated at 37°C for a time period between 24-144h before harvesting depending on the experiment. (The protocol suggests harvesting after >= 48h.)

3.4 RNA isolation

The procedure was performed according to Invitrogen's Life technologies protocol. Cells were washed with PBS and harvested by homogenization with 1ml Trizol® reagent* that was added to each well and left incubating for 5 min before put in 1.5ml RNAse-free tubes. 0.2ml chloroform was added to each sample and the tubes were shaken for 15s before centrifuged at 4°C, 12 000 x g for 15 min. The aqueous phase was transferred into fresh tubes, 1µl GlycoBlue™ was added and samples vortexed. 0.5ml isopropanol was added to each tube and mixed well by inverting them a number of times. Samples were incubated for 10 min at room temperature and then centrifuged at 4°C, 12 000 x g for 10 min. The supernatant was removed and 1ml 75% ethanol added. Samples were vortexed and centrifuged at 4°C, 75000 x g for 5 min. This wash step was repeated once, the supernatant was removed completely before the RNA pellets were dissolved in 20µl RNAse-free water. In the final step, RNA samples were incubated at 58°C, for 10 min. The total RNA concentration was measured by spectrophotometer (NanoDrop). After RNA extraction, the quality of the RNA was verified by running the samples in a 1% agarose gel. 0.4µg RNA was loaded and samples run for 40-45 min at 120 V. RNA samples prepared for Microarray analysis were purified on RNeasy spin columns (Qiagen, Hilden, Germany) according to the manufacturers protocol. The purity and amount of the samples was evaluated by NanoDrop.

*TRIZOL® Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate (www.invitrogen.com, 080130).

¤GlycoBlue is a blue dye covalently linked to glycogen, a branched chain carbohydrate. It precipitates with nucleic acids, facilitating RNA recovery while increasing the size and visibility of the RNA pellet. GlycoBlue was added to a final concentration of 50-150 µg/ml (www.ambion.com, 080130).

3.5 cDNA synthesis

The cDNA synthesis was performed using between 0.4-1.0µg of the isolated RNA. The RNA was diluted with RNase-free water to a final volume of 10µl in either strip or plate wells. The cDNA reverse transcription kit was supplied by Applied Biosystems (Foster City CA 94404, USA). A mastermix of the reagents was prepared for the samples with the following amounts of reagents: 2.0µl 10x RT Buffer, 0.8µl 25x dNTP Mix (100mM), 2.0µl 10x RT Random Primers, 1µl MultiScribe™ Reverse Transcriptase, 4.2µl Nuclease-free H_2O per reaction. In total, 10µl mastermix was added to each cDNA reaction providing a final volume of 20µl cDNA. During the procedure and until performing the reverse transcription in the thermal cycler samples were kept on ice. Thermal cycle conditions used in the program were as follows: Step 1: 25°C, 10 min Step 2: 37°C, 120 min Step 3:

85°C, 5 sec Step 4: 4ºC until put on ice or in -70°C. The system used was Gene Amp PCR System 9700 (Applied Biosystems, USA).

3.6 RT-qPCR

3.6.1 TaqMan ® Gene Expression Assays

For the detection of *WIF1*-, *GAPDH*-, *18S*- and *ALP*-transcripts, TaqMan ® Gene Expression Assays were used*. (Applied Biosystems, Foster City CA). To prepare a TaqMan® RT-qPCR run, 9µl cDNA/well was put in a MicroAmp™ optical 96-well reaction plate (Applied Biosystems). A mastermix for the plate was prepared by mixing 1µl assay and 10µl TaqMan® Mastermix (Applied Biosystems, Branchburg, New Jersey, USA) per reaction. Each reaction was run in a final volume of 20µl/well with 0.04 – 0.1µg of total cDNA. The thermal cycle program was run under the following conditions: 50°C, 2 min; 95°C, 10 min; and 40 cycles 95°C, 15 sec; 60°C, 1 min. In order to be able to detect *WIF1* in certain cases, the cycle number was extended to 45 cycles. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *18S* were used as reference genes and for each individual the absolute expression level of each transcript was normalized relative to *GAPDH*- or *18S*-expression.

*Hu *ALP* Assay ID: HS 01029140_g1, fragment length: 128.
Hu *GAPDH* Assay ID: HS 02758991_g1, fragment length: 93.
Hu *WIF1* Assay ID: HS 01548028_m1, fragment length: 66´.
Hu 18S Assay ID: HS 99999901_s1.
The pre-formulated assay (20x) mix contains 2 unlabeled PCR primers (900 nM each final concentration).
1 FAM™ dye labelled TaqMan ® MGB probe (250nM final concentration).

3.6.2 BIORAD

For the detection of *Beta actin* and all in-house designed primer pairs, reactions were run in the BIORAD iCycler system. Subsequently, 0.04 – 0.1µg of the cDNA was amplified using SYBR Green PCR master mix (Applied Biosystems, USA) under the following conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles consisting of 15 s at 94°C and one minute at 60°C. Expression values were calculated from analysis of values within the exponential phase of the amplification curve. Software system used was MyiQ.

3.7 Gel Electrophoresis

Validation of the RNA quality of isolated samples as well as evaluation of PCR-products was performed in 1% Agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving 0.5g agarose in 50ml 1xTAE buffer and 2,5µg EtBr. Loading dye was added to each sample before preparing the gel for running. The electrophoresis was run in 1xTAE running buffer at 120 V for 40 minutes and documented with an UV-camera.

3.8 Fluorometric Cytotoxicity Assay (FMCA)

Cell viability was measured with FMCA. Cells (20'000 cells/well) were seeded in 96-well plates, the day prior to transfection with MATra, introducing siRNAs. FMCA was carried out the day after transfection according to the manufacturers' instructions.

3.9 Description of experiments

3.9.1 Primer design and evaluation

Primers for osteoblast gene markers were designed at http://frodo.wi.mit.edu/ and evaluated against cDNA and genomic libraries at Ensemble (www.ensemble.org). Further, the primers were quality tested for annealing temperature and hybridization properties in the program Oligoanalyzer. 17 primer pairs (Appendix 1) were ordered from Thermo Scientific, Ulm, Germany. Efficiency evaluation of the of the received primer pairs was performed by RT-qPCR with the BIORAD iCycler System. The material used was pooled, high quality cDNA from earlier RNA extraction, prepared in a 96-well Eurogentech qPCR plate. A titration of the cDNA of the following dilutions; 5x, 25x and 125x made measurement of the efficiency from standard curve analysis.

3.9.2 Time course and dose-response experiment of BMP-2 stimulation

Before stimulation by BMP-2, cells were cultured and starved in 24-well plates, 50'000 cells/well for 24h in 0.5% FBS medium. Upon stimulation, medium was changed to 5% FBS medium. For the time course experiment cells were stimulated at a concentration of 300ng/ml, in duplicates, and harvested respectively after one day, four days and six days. Stimulation of cells harvested after six days was repeated day four after the first stimulation. For the titration experiment, cells were treated with BMP-2 at the three concentrations 300ng/µl, 600ng/ml

and 1200ng/ml, in duplicate wells respectively during with medium change and BMP-2 addition every other day. Cells were harvested after 6 days of stimulation and osteoblast markers were evaluated using RT-qPCR. Control cells were treated with corresponding amount of PBS.

3.9.4 *WIF1*-siRNA dose-response and time course experiment

In the dose-response experiment, 50000 cells/well were seeded in a 24-well plate until reached ~80% confluency at the time of transfection. The MATra transfection was carried out with three siRNAs against *WIF1* (siRNA1, siRNA2, siRNA3) and one negative control siRNA in concentrations 0.2μg; 0.35μg and 0.50μg. Cells were transfected in duplicates and harvested 72h post-transfection. As for the time course experiment, 50 000 cells/well were seeded and transfected in duplicates in a 24 well plate using all three *WIF1*-siRNAs (siRNA1, siRNA2, siRNA3) and one negative control siRNA at the concentration of 0.50μg. Cells were harvested at three time points, 24h, 48h, 72h and 144h, respectively post-transfection. RNA isolation was performed using Trizol®, cDNA was synthesized, and thereafter *WIF1* transcript levels were quantified by the TaqMan® gene expression assays and correlated against *GAPDH* transcript levels. Exactly the same procedure was performed in both MG63- and Hob-cell culture.

3.9.5 Sharp *WIF1*-siRNA experiment

250 000 cells/well were seeded and transfected with 2.5μg siRNA/well in duplicates, in 6-well plates with the three *WIF1*-siRNAs (siRNA1, siRNA2, siRNA3) and three negative control siRNAs. Cells were harvested 72h post-transfection and W*IF1* expression levels were detected as already described above.

3.9.6 *WIF1* expression in un-treated cells

In the first part, 50’000 MG63 and 200’000 Hob-cells/well were seeded in 24 well plates and grown for different time periods. Three replicate wells of each cell line were harvested at day 1, day 2, day 4 and day 6 after plating. Cells were counted and RNA was isolated at every time point. (After two days, cell culture medium was exchanged in the remaining wells. Cells were washed with PBS before new fresh medium was added.)

In the second part, 50’000, 100’000, 200’000 and 400’000 MG63 cells and Hob-cells respectively were plated in triplicate in 24-well plates, and grown for 24h before harvesting. They were counted and RNA was isolated. The RNA material was reverse transcribed to cDNA and evaluated for *WIF1* expression.

4. Results & Discussion

4.1 Summary of results

The first part of the project included experiments on primary cell culture where BMP-2 induced osteoblast differentiation was carried out. Results from these experiments showed no significant effect of the BMP-2 stimulation whereas the proceeding work was focused on the optimization of conditions for silencing of *WIF1* in primary cell culture and MG63 cell culture without BMP-2 stimulation. The optimal dosage of siRNA resulted in 0,5µg siRNA/50'000 cells in 24-well plates and the post-transfection incubation time in 72 hours in both cell cultures. Under these conditions, the effect of the silencing was up to as much as 98% decrease in *WIF1* expression.

4.2 Primer design and evaluation

The effect of BMP-2 stimulation in Hob- cell culture is usually quantified as the gene expression of known osteoblast markers. Hence, 19 different primer pairs for osteoblast marker genes were designed and evaluated using quantitative PCR (qPCR) with SYBR Green. The quality and efficiency of the primers was calculated from standard curves generated from a dilution series of every primer pair. Unfortunately, a considering part of the primer pairs (results not shown) did not fit the recommended conditions for adequate primers. The standard curve slopes and thereby the efficiency of the reactions were not sufficient and only one of the primer pairs, for *ALP* is illustrated in the following section. This outcome is not unusual, since there are several difficulties designing proper primers and the design and optimization procedure includes a number of steps where it is important to be aware of possible pitfalls.

The amplification plot and melt curve for three dilutions of *ALP* primers prove good efficiency with a standard curve slope of -3,209 (recommended interval is -3,6>=-3,1). The melt curves imply only one product in the sample with a melt temperature of 86°C and there are no indications of primer dimmers (Figures 5 and 6) An example of a less efficient amplification is the one for *SFRP1* where the amplification plot and melt curve are not optimal but still show relatively good efficiency with a standard curve slope of -2,964.

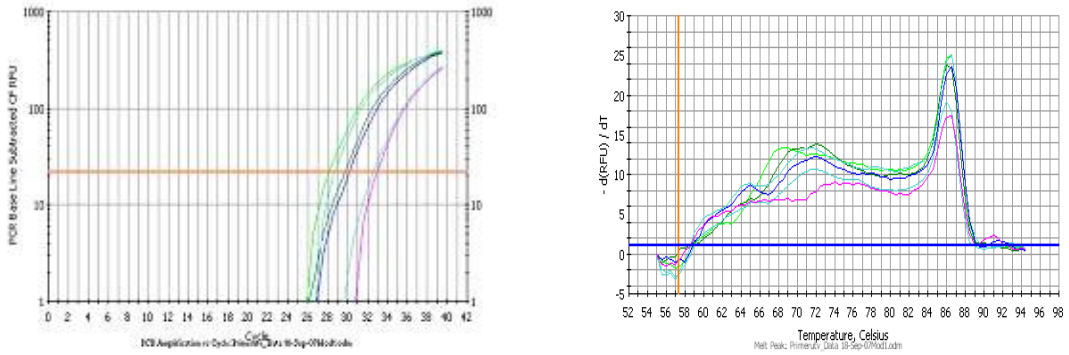

Figure 5: Amplification plot (a) with SYBR Green for *ALP* primer pairs dilutions (a five fold cDNA concentration difference between the samples). The three different primer dilutions are clearly separated and visualized in the graph window even if there is a noticeable difference between the replicate samples. Melt curves (b) for *ALP* confirming the melt temperature of the product at 86°C.

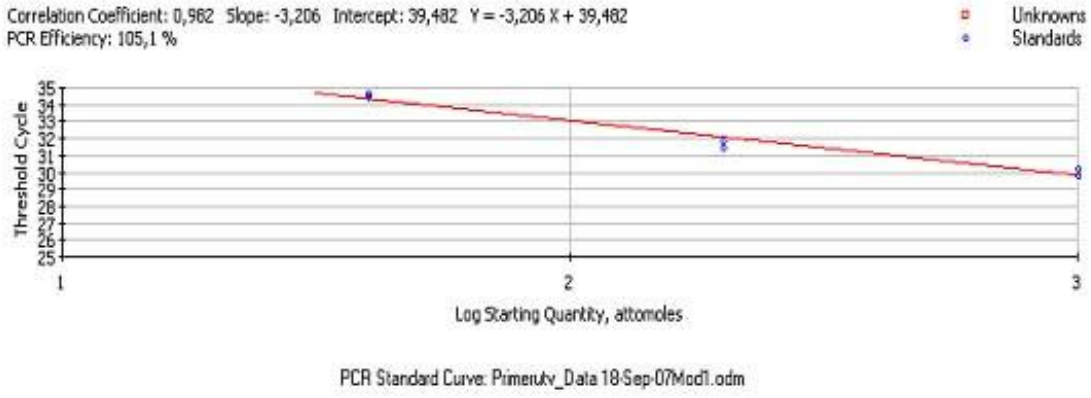

Figure 6: Standard curve of three *ALP* primer dilutions. The curve slope was calculated to -3,206 and the efficiency of the reaction 105,1%.

4.3 Time course and dose-response experiment of BMP-2 stimulation

The osteoblast marker genes *ALP* and *SFRP1* were evaluated with qPCR and correlated to the reference gene, b*eta-actin* expression as a first attempt to evaluate the efficiency of the BMP-2 treatment. *ALP* was successfully amplified in the time course experiment while the contrary was seen in the dose-response experiment. *ALP* transcript levels were thus calculated and compared among BMP-2 samples and not-treated samples for the time course experiment but not the dose-response experiment (Figure 7). Similar results were obtained for *SFRP1*, i.e. the amplification was better in the time course experiment than in the dose-response experiment, though not reliable enough to do calculations comparing the transcript levels in the different samples (not shown).

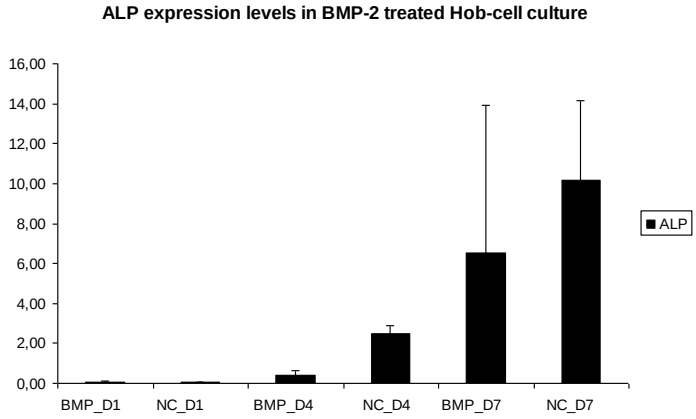

Figure 7: *ALP* transcript levels calculated from BMP-2 time course stimulation experiment and correlated to *Beta actin* transcript levels. D1 = One day after first stimulation etc and NC (negative control) meaning not treated cells. No significant difference between NC samples and BMP-2 treated cells is seen.

The poor amplification shown for *ALP* in the BMP-2 dose-response experiment is probably due to poor quality RNA generated during RNA isolation. The gel electrophoresis showed very poor bands for RNA. Therefore it was simply too little RNA in order to attain an effective PCR reaction for *ALP*. Beta actin on the other hand was successfully amplified, which is probably due to the natural abundance of the mRNA transcript. Then only a small RNA quantity makes it possible to amplify b*eta-actin*. Noteworthy is the difficulty to handle this type of cells (Hob- cells) and their low growth rate. Since they grow very slowly the RNA yield was low in all samples, which makes it delicate to handle. It is definitely an advantage to be experienced with working with this specific cell line to achieve good results.

4.4 MATra based Transfection with siRNA against *WIF1* in Hob- and MG63 cell culture

Magnet Assisted Transfection Assay (MATra) which is the effective method to introduce nucleic acids to cells was used with the aim to achieve at least 65% decreased expression levels of the target gene, *WIF1*. Therefore the required optimal siRNA quantity was first determined in a dose-response experiment, followed by a time course experiment finding out the consistency of the silencing effect. Results from these experiments and follow-up experiments are described in the following section where transcript levels were measured by absolute quantification with TaqMan gene expression assays and correlated to *GAPDH-*, *beta-actin-*, or *18S* transcript levels.

4.4.1 WIF1-siRNA dose-response experiment,

Three different siRNAs (siRNA1, 2 and 3) directed to *WIF1* transcripts and one scrambled siRNA (negative control) were introduced in three doses (0,2µg; 0,35µg and 0,5µg) in the range of the manufacturer's protocol suggestions. MATra-transfection was performed the day after plating 50'000 cells/well in 24-well plates, and cells were harvested 72h post transfection. This procedure was identically performed in MG63 cell culture and Hob- cell culture. In MG63 cells, the silencing effect was augmented with increased amount of siRNA (Figure 8). As for Hob- cells, a good silencing effect is visible even though negative control siRNA samples exhibit a large variation (Fig 5). Results concerning MG63 were satisfying and the outcome was that the higher the dose of siRNA cells were treated with, the greater silencing effect of the target gene, *WIF1*. This pattern is well depicted in Figure 4, where the three doses 0.2µg; 0.35µg and 0.5µg siRNA all represent a knock-down in *WIF1* transcript levels but where 0,5µg siRNA has the superior effect. It appears that 0.5µg siRNA1 is able to decrease *WIF1* levels to un-detectable levels. The RNA yield and quality are comparable and high-quality in all samples. Furthermore the reference gene transcript levels have no significant fluctuations between negative control samples and *WIF1*-siRNA treated samples, and thus a reliable quantification can be done.

The dose-response experiment in Hob-cell culture (Appendix 2) does show a great variance between biological replicates, which probably is due to the natural variance in the cell material. Even though cells from the same origin at the same passage are used, it may be that they respond to the treatment during the experiments in different ways. One could also suspect that a mix-up between the siRNAs '0.5µg siRNA3' and '0.5µg siNC has taken place since *WIF1* transcript levels are higher in the samples treated with siRNA3 than in those treated with siNC. Anyway, we can be sure that there is an effect on *WIF1* by the treatment also in Hob-cell culture.

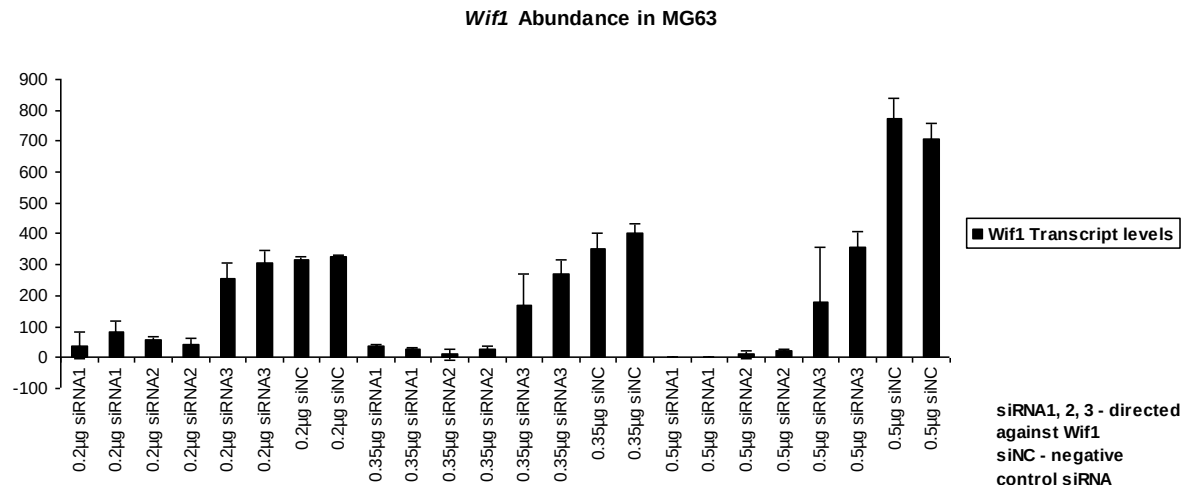

Figure 8: MATra transfection in MG63 cell culture, cells harvested 72h post transfection with three siRNA concentrations, 0,2µg; 0,35µg and 0,5µg. *WIF1* transcript levels measured by TaqMan Gene Expression Assays are correlated to the reference gene *GAPDH* transcript levels.

4.4.2 *WIF1*-siRNA time course experiment

MATra-transfected cells with siRNA1, 2, 3 and one negative control siRNA were harvested at four time points, 24h, 48h, 72h and 144h post transfection and evaluated for *WIF1* transcript levels. 50'000 MG63 cells/well were transfected in 24-well plates. The effect of silencing in this experiment was in between 42% (24h post-transfection incubation) to 77 % (72h post-transfection incubation) where the effect is clearly increased with time (Figure 9).

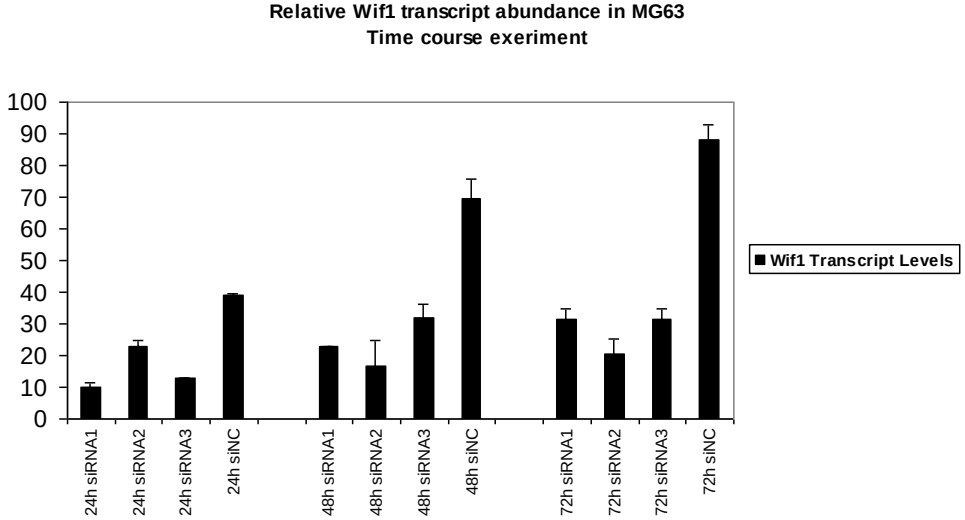

Figure 9: *WIF1* transcript levels correlated to *GAPDH* expression at time points 24h, 48h and 72h post transfection with 0,5µg siRNA in MG63 cell culture.

Interestingly, 144 hours after transfection, transcript levels of *WIF1* in MG63 cells are extensively higher than after 72 hours (Appendix 2). It seems that the longer time in culture, the higher *WIF1* expression. This is true for cells treated only with negative control siRNA. Since little yet is known about *WIF1* and its effects, it was interesting to perform another type of experiment, to investigate why the transcript levels are so much higher after six days in culture. It was hypothesized that *WIF1* transcript levels in cells is dependent either on the time period cells spent in culture or the actual cell number (how crowded the dishes/plates are). The follow-up experiment is described in section 4.5.

4.4.3 Sharp *WIF1*-siRNA experiment

In order to attain greater RNA yield and to be able to assess variance among negative control siRNAs, 250 000 cells/well were seeded and transfected with 2.5µg siRNA/well in 6-well plates with the three *WIF1*-siRNAs (siRNA1, siRNA2, siRNA3) and three negative control siRNAs. To further examine whether there was any differences in results depending on the reference gene chosen for the experiments, *WIF1* was correlated to both *18S* and b*eta-actin*. This experiment was also performed in both MG63- and Hob-cell culture.

The estimated efficiency of the respective siRNA1, 2 and 3 in Hob- cell culture was 93,6 %; 80,9 %; and 85,8% as *WIF1* was correlated to the *18S* reference gene (Figure 10). When correlated to b*eta-actin* efficiencies of 93,9 %; 80,0 % and 87,4 % were observed (Appendix 3). The corresponding figures for MG63 were 80,0 %; 93,3 % and 79,0 % correlated to *18S* (Figure 11) and 75,7 %; 90,3 % and 75,9 % when correlated to b*eta-actin* (Appendix 3). The results are comparable showing that both *18S* and b*eta-actin* reference genes function as good reference genes in calculating *WIF1* transcript levels. Further using *GAPDH* as reference gene gave similar results, indicating that either of the three can be used in this kind of experiment. Thus, results show great variance in *WIF1* transcript levels between the three samples treated with negative control (NC)siRNA (Figure 10-11). In MG63 cell culture, the original NCsiRNA has the same appearance as in previous experiments but the two new ones differ from the first. They appear to have a suppressive effect on *WIF1* transcripts even though they should not have any effect at all. The effect could also be interpreted the other way around, that the original siRNA in that case should have stimulating effect on *WIF1* transcripts. Nevertheless the negative controls are not consistent and there is no obvious explanation for this outcome. Results from Hob-cell culture demonstrate a better correlation between negative controls and thus the results are very much as expected.

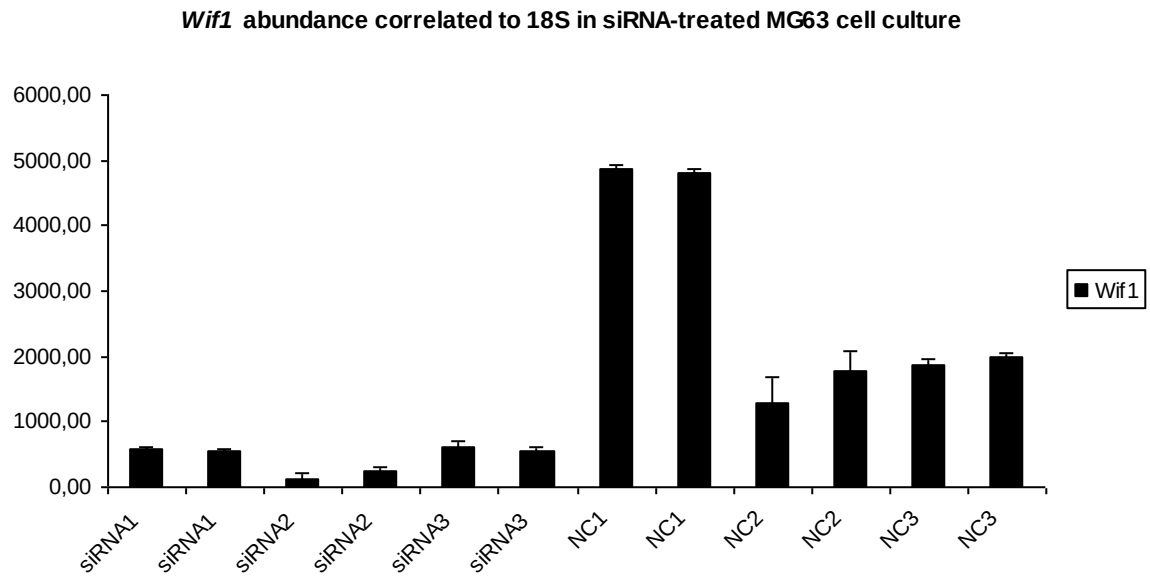

Figure 10: *WIF1*-siRNA experiment in MG63 cell culture with *WIF1* correlated to 18S transcript levels. 6-well format with three negative control siRNAs (NC1, NC2, NC3) and one MATra-control. Cells harvested 72h post transfection.

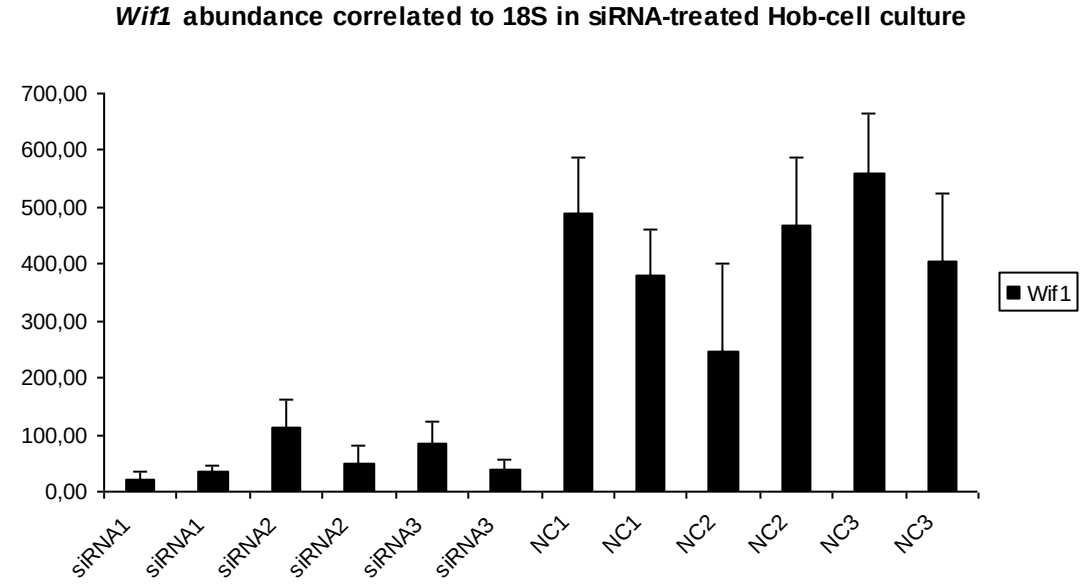

Figure 11: *WIF1*-siRNA experiment in HOB- cell culture with *WIF1* correlated to 18S transcript levels. 6-well format with three negative control siRNAs (NC1, NC2, NC3) and one MATra-control. Cells harvested 72h post transfection.

4.5 *WIF1* expression in un-treated cells

To verify if the expression of *WIF1* (interpreted from results in 3.2) is due to how many days the cells have been in culture or if it is due to the actual cell number, two experiments were performed where both human osteoblast cells and the MG63 cell line were used. Cells were counted at every time point of harvesting. In the first part, four different amounts of cells were cultured for 24h in order to assess *WIF1* levels. In the second part, an equal cell amount was seeded and cultured for a time period of 6 days and harvested at four different time points.

The relative *WIF1* transcript numbers are high in MG63. None of the samples are deviant and thus do not show higher transcript numbers than other samples. In some of the samples though, MG63 show higher transcript levels of *WIF1* than *GAPDH*, which could be due to the fact that MG63 is actually derived from osteosarcoma cancer, where it has been shown that *WIF1* expression is higher in comparison to normal cells. When it comes to Hob-cells, there is a consistency in *WIF1* transcript levels among all samples, which does not seem to depend on the actual cell number at all (Appendix 4). The number of cells was approximately the same at the time when plated and at the time for harvesting after 24h, both in MG63- and Hob-cell culture.

In the experiment leaving cells in culture for different time periods there are no evident conclusions to be drawn about *WIF1* transcript levels being dependent on the number of days in culture. In the MG63 cells, there is a tendency that *WIF1* transcript numbers are increased the longer the cells are left in culture (Appendix 4) but since the cell number is also greatly increased from one day to six one needs to take this into account. In contrast, *WIF1* transcripts in Hob-cells are decreased from day one to six (Appendix 4). This could simply be explained by the decreasing cell number at each time point, which eas observed after counting cells at every time point of harvesting.

4.6 FMCA

Viability of MATra-transfected and non-transfected cells was measured in one single experiment in 96-well plates (20’000 cells/well). The FMCA measurement was performed one day post-transfection and the results show no significant difference in viability of transfected cells in comparison to non-transfected cells, indicating that the transfection method is not toxic for the cells (data not shown).

5. Conclusions & Future Perspectives

Results from this study demonstrate successful silencing of a novel candidate gene for bone metabolism, *WIF1*, in *vitro*. Silencing was performed introducing *WIF1* - sequence specific siRNAs into cells by Magnet Assisted Transfection (MATra). It was possible to knock-down *WIF1* transcripts both in primary human osteoblast cells (Hob-cells) and in the MG63 osteoblastic cell line with up to 98 % efficiency. In order to reach these levels of silencing, the siRNA dosage and post-transfection incubation time were optimised. As an appropriate silencing effect is defined as a 65 % decrease in transcript levels of the knocked-down gene, the results are very promising. Noteworthy is that when using RNAi methodology with siRNAs, one strives to attain knocked-down expression of a target gene, rather than knock-out. The difference from knock-out experiments is that we still want to be able to detect our target gene expression and quantify the actual knock-down effect compared to negative control samples. This would not possible in knock-out experiments.

5.1 WIF1

WIF1 was chosen as target gene since it is hypothesized to play an essential role in bone metabolism. As mentioned earlier in the report, the gene is differentially expressed between two chicken strains, and Single Nucleotide Polymorphisms (SNPs) within the gene have been significantly associated with bone phenotypes in a human cohort (preliminary results, data not shown). *WIF1* is known to be an inhibitor of factors in the *Wnt* signalling system, implying that the knock-down could have an effect on bone metabolism. Results from this master thesis on *WIF1* forms a basis for further research on other novel candidate genes but also warrants further follow-up experiments concerning *WIF1*. The next step in the procedure will be global microarray-analysis of the now existing knocked-down RNA material, to get an indication of what transcriptional effects knock-down of *WIF1* may comprise. Probable outcomes are that certain genes or pathways would be either up- or down-regulated as a result of *WIF1* knock-down. These up- or down-regulated genes of interest will thereafter be analysed by quantitative PCR in actual transcript levels from the original RNA material, making a comparison between knocked-down and negative control RNA. If the verification agrees with the results from the microarray analysis, one will probably have found genes/pathways that are affected by the knock-down experiment. Another interesting experiment for the future is to verify the silencing effect on translational level, i.e., to study *WIF1* protein levels in siRNA treated cells compared to un-treated cells. Furthermore, overexpression (vector based) of the interesting gene product is another step in the characterization of novel genes. Additionally, it is possible to load recombinant WIF1 protein into cells

to further characterize the cell response to this overdose. In order to employ protein stuies in the laboratory, a good protein detection system is needed.

5.2 BMP-2

WIF1 normally is a low-expressed gene in the cell, and the first attempt in this project work was to increase the production of *WIF1* by induction of cell differentiation using BMP-2. It was shown by Vaes et al. (2005) that *WIF1* expression levels are higher in late-phase differentiated cells after BMP-2 induction in C2C12 and MC3T3 cells. In the same study *WIF1* was found to be expressed in vivo, in trabecular but not in cortical bone. Since the cellular model in this project consists of trabecular bone derived cells, this finding provide evidence that the model may be even more advantageous for purposes of detecting *WIF1* than originally thought. Since we were unable to confirm the results by Vaes we decided to examine if the natural *WIF1* expression levels were sufficient to be detected by TaqMan expression assay without BMP-2 stimulation. Fortunately, this was possible, in all non-treated Hob-cell samples, *WIF1* was detectable, and so the siRNA experiments were started. Even though the differentiation induction with BMP-2 did not provide the expected results, it could be of value to do iterative experiments of this kind. Since primary cell cultures can be difficult to work with and there is a possibility there are other cells than only osteoblasts in this case, BMP-2 stimulation is a way to establish a more reliable cell culture, containing late phase osteoblasts.

5.3 Cells and FMCA

Primary cells usually grow slowly in comparison to cell lines. Therefore the Hob-cell proliferation rate is much lower than for MG63. During three days of incubation after treatment with MATra transfection, the Hob-cells did not proliferate at all while MG63 increased in cell amount with 100 %. MG63 cells are overall very resistant and seem not to be affected by the harsh treatment during MATra transfection. One possible hypothesis when it comes to Hob-cells could then be that Hob-cells are more sensitive to and therefore respond to a higher degree to this treatment. It is possible that they come to the stop in their proliferation just after MATra treatment and thereby become hindered in their proliferation. The cells are clearly viable, and it is possible that the proliferation pace can return after sufficient time in culture and with the right nutrient supply. To confirm the good shape of cells after a treatment like this, a method called semi-automated fluorometric microculture cytotoxicity assay (FMCA) was used. This methodology allows the measurement of the viability in cell culture. A small-scale experiment was prepared in the laboratory, both for Hob-cells and MG63, when

measuring viability with FMCA in cell cultures, MATra transfected with *WIF1*- and NC-siRNA in 96-well format (results not shown), which showed no significant cell death after treatment. It should be of interests to extend these assays in the future and perform FMCA on cells transfected in 24- and 6-well format as well to be able to use the results together with the actual silencing experiments. In the future it will be of greater interest to do research on human primary bone cells rather than MG63 cells, but MG63 cells may function as a reference, as these cells are very robust in culture. The FMCA is an important technique in these kinds of studies since it is of importance to know that cells survive the actual treatment, i.e. in this case the MATra treatment could possibly be lethal to the cells.

One experience from this study is that it is not easy designing fully functional primers. There are many factors to think of and pitfalls to avoid. For example, when generating the sequence, it may be that perfect base pairing is difficult to achieve, or there can be several sites possible for primer pairing to choose from. The optimal case where primers overlap two exons can also be difficult to accomplish. Furthermore the properties of the primer sequences matters, for example high G/C content increases the risk for mis-priming but instead facilitates tight binding to the template. Many of the designed primers are G/C rich and thus the possibility for mis-priming could have caused the poor efficiency. It is recommended that primers should end 3' in a C or G, CG or even GC to prevent a unfavourable binding of ends and to increase efficiency of priming. The above-mentioned factors are probably contributory to the poor efficiency of some of the primers designed within this study.

5.4 Bone genetics in Uppsala

The bone genetics group in Uppsala has a unique research position within the field of functional genomics of bone, since it is one of the few laboratories in the world that obtains primary bone cells for research purposes directly from patients. Cooperation with the orthopaedic department at Uppsala University Hospital facilitates this tissue sampling and creates opportunities, for the research group, to gain a position in the front of the research field of bone genetics in the near future. In general, most cell culture experiments are performed on commercially available cell lines, and there are many advantages to use human primary cell culture for research purposes. The MATra-technique was introduced recently to the Uppsala laboratory, and has now been shown to be effective for primary osteoblast transfection as an alternative to electroporation. Since transfection of primary cells is not easy it was a good opportunity to be able to apply it in this master's degree project. Electroporation, which earlier was employed as transfection method in the laboratory, is known to be harsh to cells since it disrupts cell membranes during the process allowing siRNAs to enter before membranes they

are closed again. MATra is claimed to be less harmful to the cells since it applies magnetic forces that pulls the nucleic acids associated to the magnetic beads into the cells. Since the MATra technique is successful on this type of cells it has the potential to become a very useful tool among others, a tool that is both easy to handle and relatively quick to perform.

In summary, this master thesis work has successfully shown that gene silencing of novel candidate genes for bone metabolism can be performed in primary bone cells and bone cell lines. Several techniques novel to the laboratory were introduced during the master thesis work, e.g. Magnet Assisted Transfection, and Fluorometric Microculture Cytotoxicity Assay (FMCA), and were employed in studies of the promising candidate gene *WIF1*. Currently, RNA from the master thesis work awaits global mRNA expression analysis. There are yet further interesting bone-related genes to study, and based on the findings of this master thesis, there are relevant cell models and genomic tools to use for studies of these candidate genes.

6. Acknowledgements

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Appendix 1 – RT-qPCR Primers

SFRP1_1
Left primer: CGAGTTTGCACTGAGGATGA
Right primer: CCCCATTCTTCAGGTACAGC

SFRP1_2
Left primer: TTTGAGGAGAGCACCCTAGGC
Right primer:TGTGTATCTGCTGGCAACAGG

SFRP2
Left primer: GGCCTGCAAAACTAAAAACG
Right primer: TTGCTCTTGGTCTCCAGGAT

RUNX2_1
Left primer: TAGGCGCATTTCAGATGATG
Right primer: CACCTGCCTGGCTCTTCTTA

RUNX2_2
Left primer: CCAACCCACGAATGCACTATC
Right primer: TAGTGAGTGGTGGCGGACATAC

SP7 (Osterix/Osx)
Left primer: CCCCAGGAGGAAGTTCACTA
Right primer: CTGCTTTGCCCAGAGTTGTT

IBSP_1 (Bsp)
Left primer: GACTGCCAGAGGAAGCAATC
Right primer: TTGAGAAAGCACAGGCCATT

IBSP_2
Left primer: TCTGAAGAAAATGGGGTCTTT
Right primer: CACTACTGCCCTGAACTGGA

DLX5
Left primer: CCAACCAGCCAGAGAAAGAAG
Right primer: TCCTGGGTTTACGAACTTTCT

LRP5:1
Left primer: GGAGCGAGGGTACCTGTACTT
Right primer: AGTGTGTTGTCCACCACCAG

LRP5:2
Left primer: TGCTCCCACATCTGTATTGC
Right primer: CTCTCCACAGGTCAGCAGGT

FZD1
Left primer: AGCCGAGAAAGTATGGCTGA
Right primer: AGCGTAGCTCTTGCAGCTCT

LEF1:2
OLIGO
Left primer: GGCTGGTCTGCAAGAGACA
Right primer: TCGTTTTCCACCATGTTTCA

WIF1:1
Left primer: ACCCCACGATGTATGAATGG
Right primer: TGGTTGAGCAGTTTGCTTTG

WIF1:2
Left primer: ACAACCCTGTCGAAATGGAG
Right primer: GCAGACAGGCTTTGAACAGA

ALP:1
Left primer: AGCACTCCCACTTCATCTGG
Right primer: TGGCTCGAAGAGACCCAATA

ALP:2
Left primer: GCCCTCTCCAAGACGTACAA
Right primer: GCTCAGTGGCTGCGCTTAC

Appendix 2 – Results, *Wif1* silencing

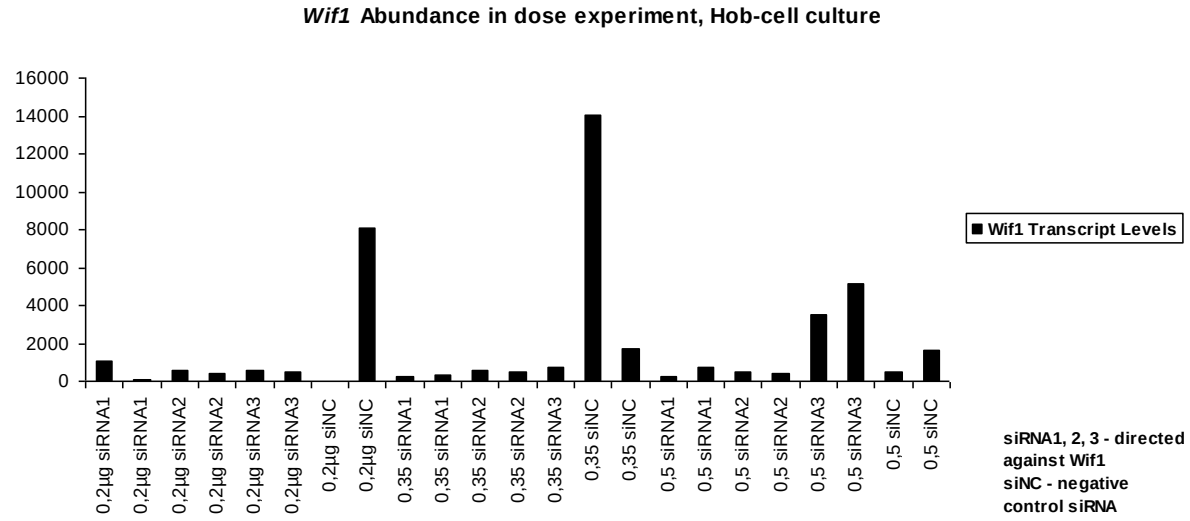

Figure 5: MATra transfection in Hob-614 cell culture, cells harvested 72h post transfection at three different siRNA concentrations, 0,2µg; 0,35µg and 0,5µg. *WIF1* transcript levels are correlated to the reference gene *GAPDH* transcript levels.

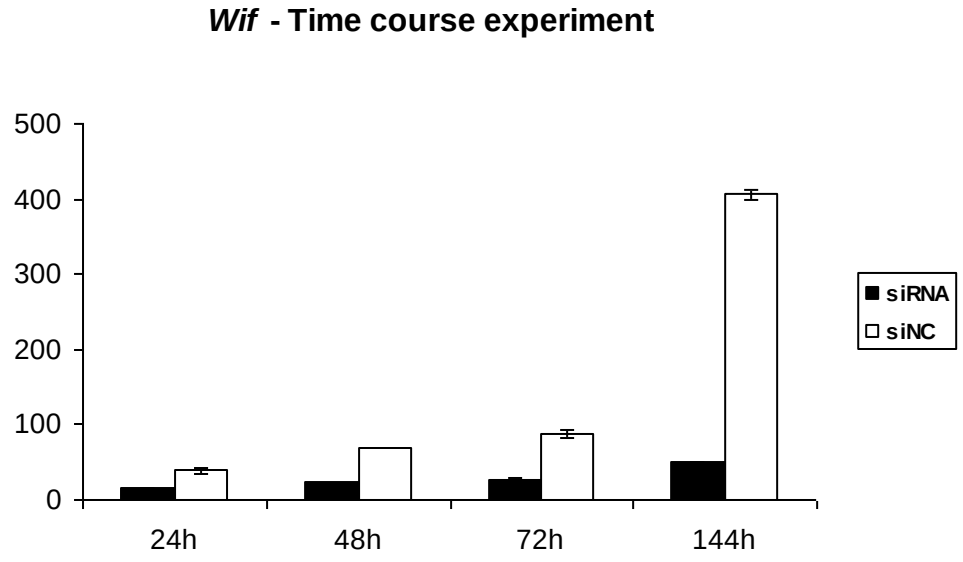

Figure 6: Visualization of *WIF1* transcript levels in the time course experiment showing the major increase in *WIF1* transcripts after the time period 144h after transfection, in cells treated with negative control siRNAs in MG63 cell culture.

Appendix 3 – Results, *Wif1* correlated to Beta actin

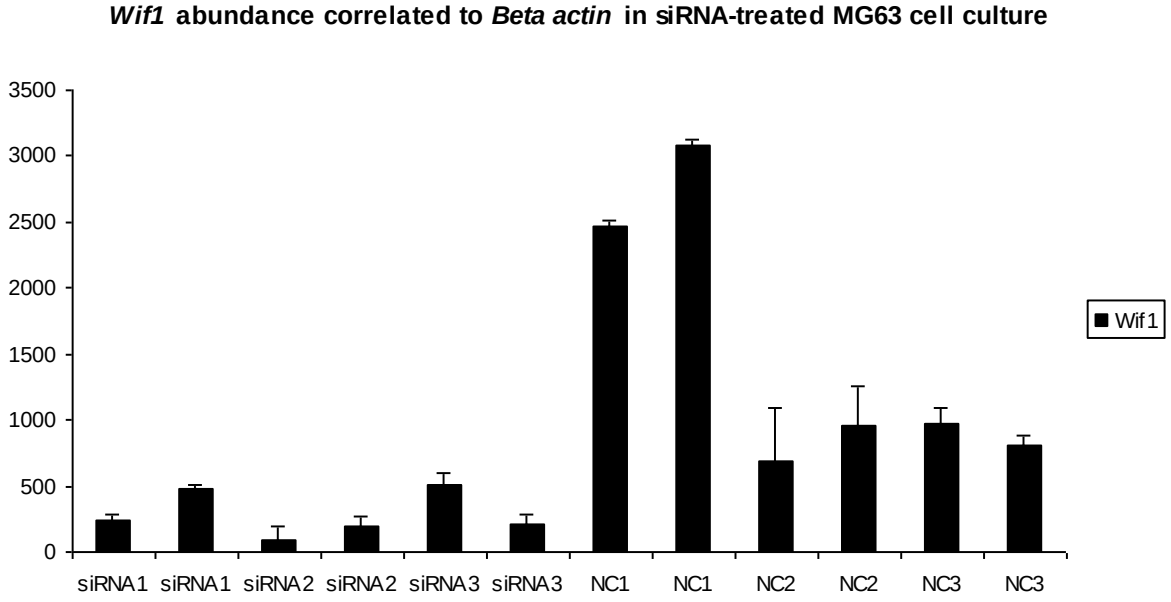

Figure 8: *WIF1*-siRNA experiment in MG63 cell culture with *WIF1* correlated to Beta actin transcript levels. 6-well format with three negative control siRNAs (NC1, NC2, NC3) and one MATra-control. Cells harvested 72h post transfection.

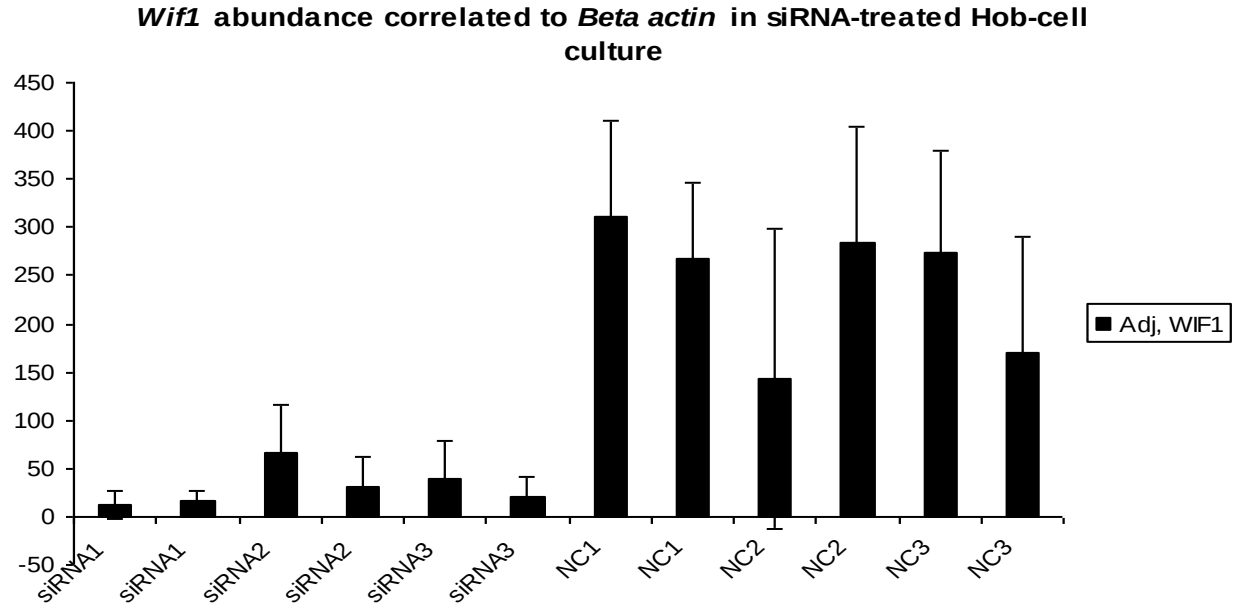

Figure 10*: WIF1*-siRNA experiment in HOB- cell culture with *WIF1* correlated to Beta actin transcript levels. 6-well format with three negative control siRNAs (NC1, NC2, NC3) and one MATra-control. Cells harvested 72h post transfection.

Appendix 4 – Results, *Wif1* in un-treated cells

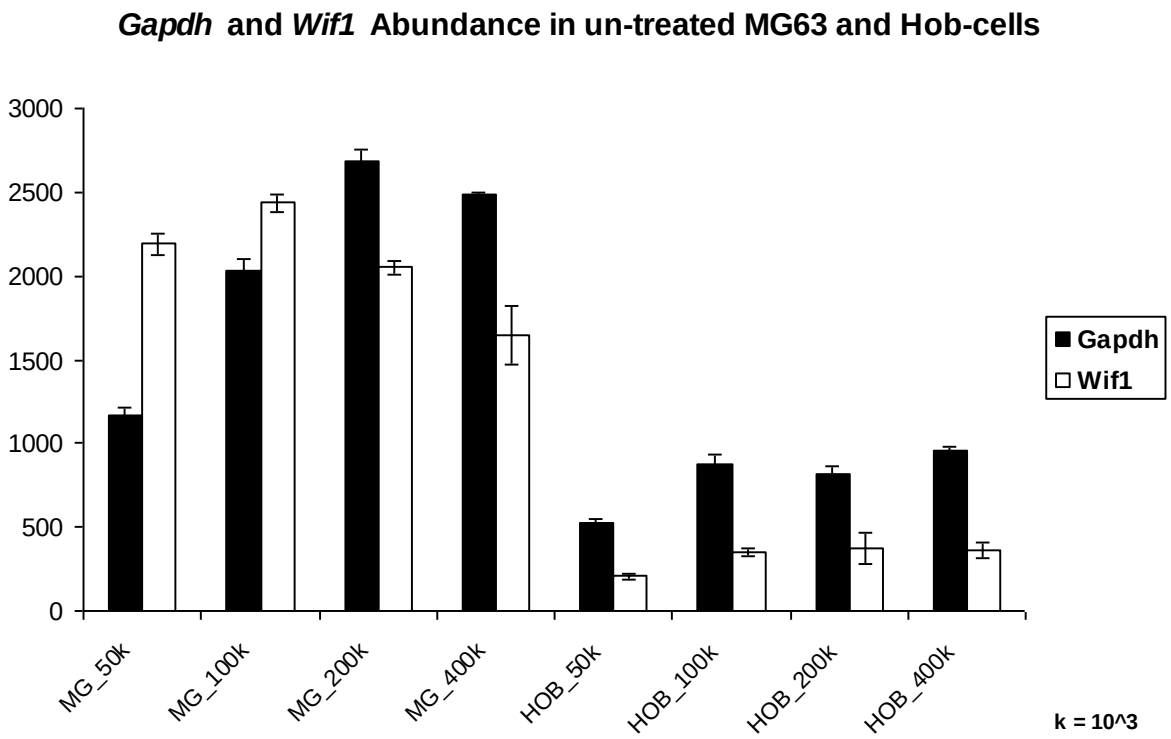

Figure 11: *WIF1* and *GAPDH* transcript levels in untreated MG63 and Hob- cell cultures plated in four different amounts, 50'000, 100'000, 200'000 and 400'000 cells, and harvested the day after plating.

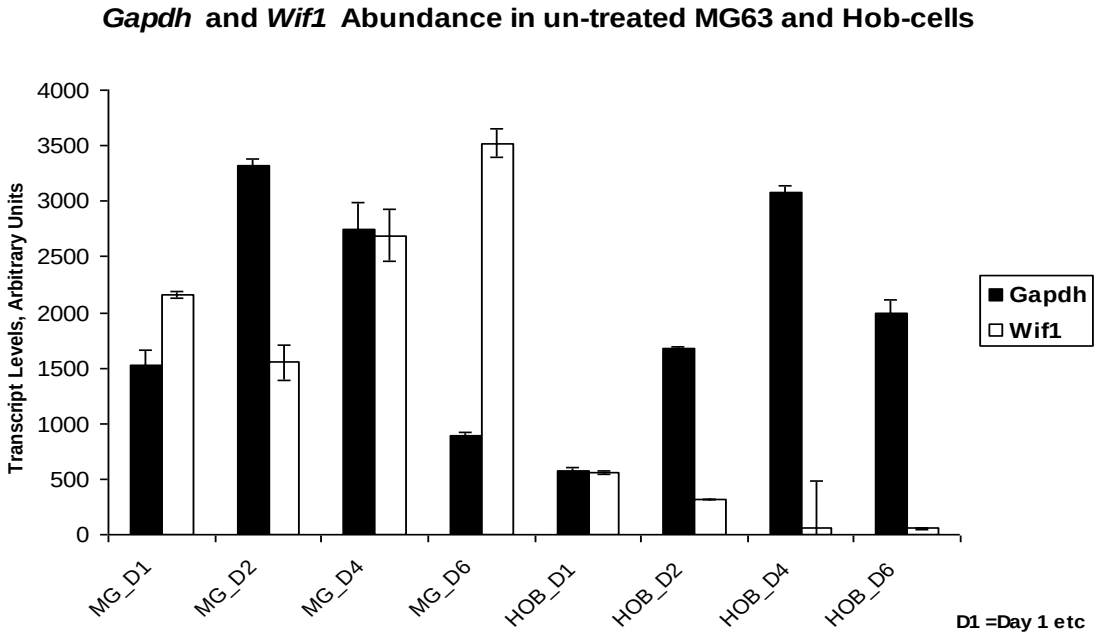

Figure 12: *WIF1* and *GAPDH* transcript levels in untreated MG63 and Hob- cell cultures, 50'000 cells/well, harvested respectively at day 1, 2, 4 and 6 after plating.