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"THE ROLE OF SMAD UBIQUITIN REGULATORY FACTOR 1: SMURF1 IN THE DIFFERENTIATION 3T3-L1"

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Abstract

The Smad Ubiquitin Regulatory Factor-1: SMURF1 is an E3 ligase. This E3 ligase has been linked to several important biological pathways, including the bone morphogenetic protein pathway, the non-canonical Wnt pathway and the mitogen-activated protein kinase pathway. Multiple functions of Smurf1 have been discovered in cell growth and morphogenesis, cell migration, cell polarity and autophagy. Previous studies, in 2003, have demonstrated that overexpression of Smurf1 induces proteasomal degradation of Smad1 and Runx2 proteins.

Runx-2 is a transcription factor that is essential for osteoblastic differentiation, bone formation and maintenance. Both the pre-adipocyte and osteoblast cells are originated from common mesenchymal progenitor cells and it has been recently demonstrated that Runx2 is also involved in adipocyte differentiation.

The major goal of this project is the study the role which Smurf1 plays in the differentiation of 3T3L1 cells.

The abbreviations used are:

1. SMURF1: Smad Ubiquitin Regulatory Factor-1
2. BMP: Bone Morphogenetic Protein
3. E3: Ubiquitin Ligase
4. Smad: Sma/Mother against *dpp*
5. Runx2: Runt Domain Transcription Factor-2
6. TGF-β: Transforming Growth Factor β
7. FBS: Fetal Bovine Serum
8. DBS: Donor Bovine Serum
9. DMEM: Dulbecco´s modified Eagle´s

Introduction

Smurf1

The Smad Ubiquitin Regulatory Factor-1 (Smurf1) is a protein which participates in processes like cell growth control, cell morphogenesis, cell migration, cell polarity and cell autophagy. Smurf1 is homologous to the E6-associated protein carboxy1 terminus (HECT) domain E3 ligase, a protein which contains a N-terminal C2 domain for membrane binding, a central region with two or three WW domains for protein-protein interaction and a C-terminal HECT domain for ubiquitin-protein ligation. Smurf1.

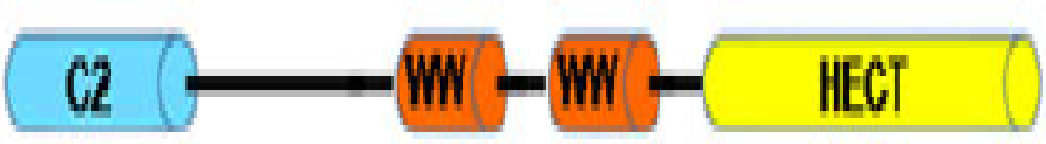


Figure 1. Smurf1 structure (Modified, Cao *et al.*, 2012).

Originally, the Smurf1 was identified an E3 ubiquitin ligase for Smad1 and Smad5, two major proteins in the Bone Morphogenetic Protein (BMP) pathway. Smurf1 promotes their ubiquitination and proteasomal degradation, and thus it down-regulates BMP signalling and osteoblast differentiation. In addition it has been shown that it also participates in another pathways such as the non-canonical Wnt pathway and the mitogen-activated protein kinase pathway. Zhao´s group demonstrated in 2004 that Smurf1 inhibits *in vitro* osteoblast differentiation and *in vivo* bone formation (Zhao *et al.*, 2004).

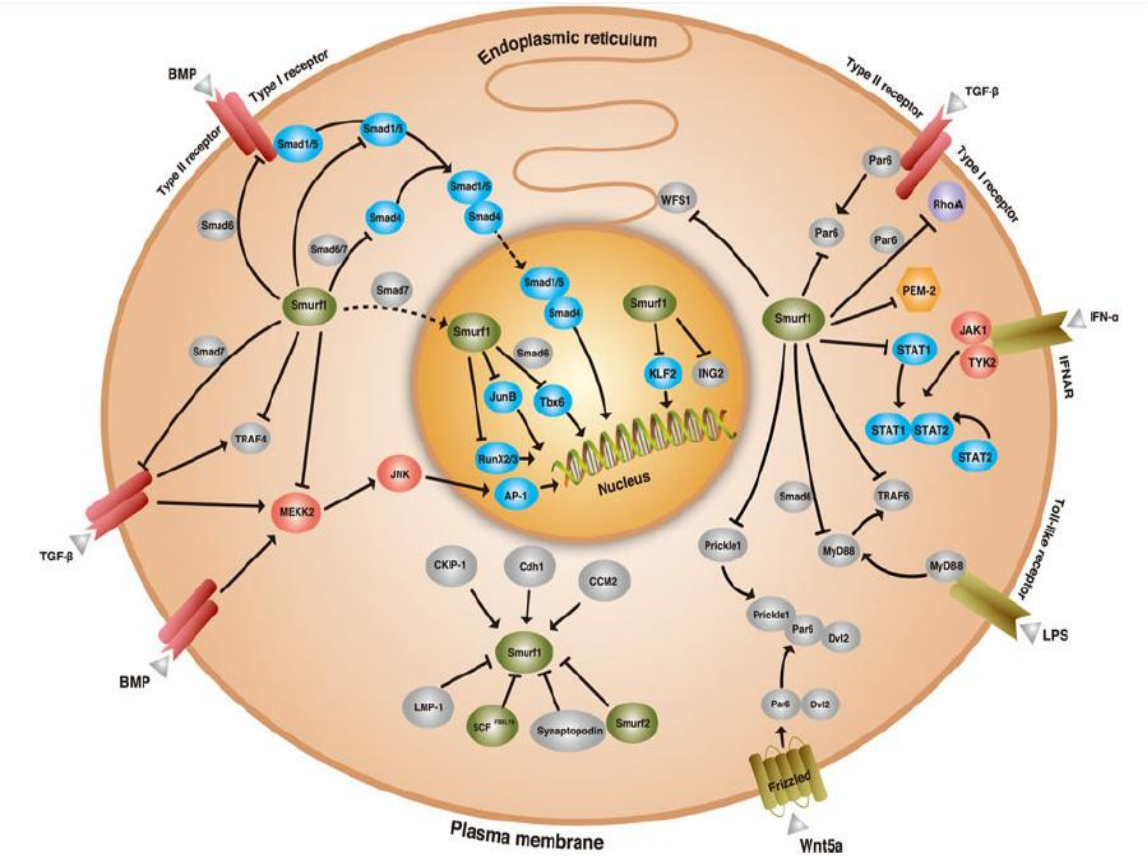


Figure 2. Role of Smurf1 in multiple biological networks (Cao *et al*., 2012).

In addition to Smad1 degradation Smurf1 also induces the degradation Runx2 although both are done by independent mechanisms (Fig 3) (Shen *et al*., 2006).

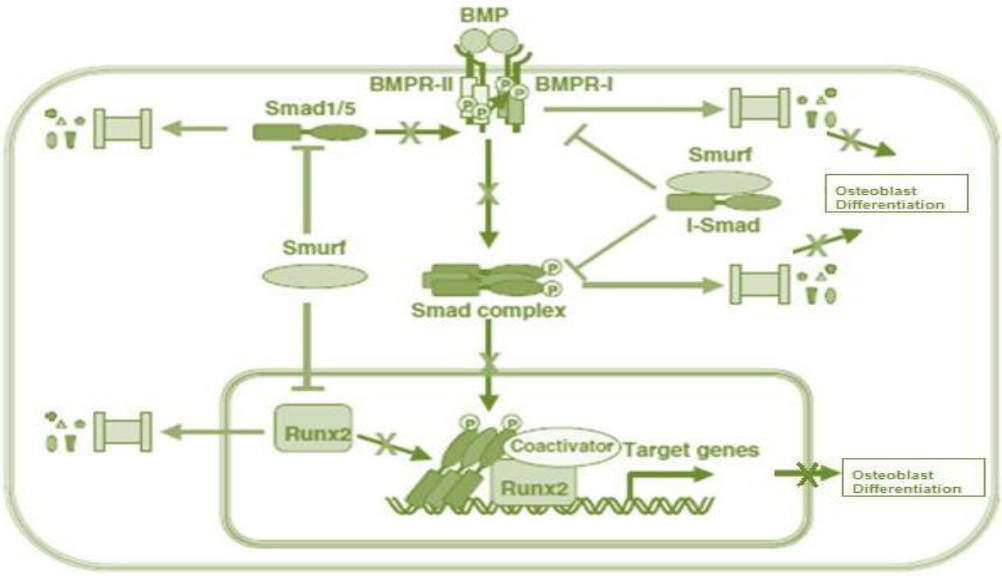


Figure 3. Runx-2 and Smad1 degradation (Modified Miyazono *et al.*, 2004).

Runx-2

Runx-2, a member of the RUNX family and a Smurf 1 target, is an important transcription factor that regulates different biological processes. It is essential for osteoblast differentiation, bone formation and maintenance. It is activated during bone formation and it regulates the expression of osteoblast marker genes such as osteocalcin (Liu et al., 2001). It has recently shown its involvement in adipocyte differentiation (Takahashi, 2010).

Smad1

SMADs are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream gene transcription. There are three classes of SMAD: Receptor-regulated Smads, Common-mediator Smads and Inhibitory Smads (Miyazono et al., 2004).

Smad1 is a Receptor-regulated Smad that mediates the signals of bone morphogenetic proteins (BMPs), which are involved in a range of biological activities including cell growth, apoptosis, morphogenesis, and development. This protein can be phosphorylated and activated by BMP receptor kinase, and is a target for Smurf1 (Zhao et al., 2003).

Mesenchymal cells differentiation

Several lines of evidence indicate that osteoblast, chondrocytes, myocytes and adipocytes are all derived from a common progenitor cell named mesenchymal cells.

In accordance with Student *et al.*, 1980 a differentiated cell in characterized by specialized functions which are a manifestation of a unique complement of proteins

- **Adipogenic differentiation**

  Adipocytes play a central role in lipid homeostasis and the maintenance of energy balance in vertebrate organisms. They store energy in the form of triglycerides during periods of nutritional abundance and release it in the form of free fatty acids at times of nutritional deprivation. Pathological conditions associated with altered adipocyte cell number or functions include obesity and several lipodystrophy syndromes. In humans, obesity is an independent risk factor for noninsulin-dependent *Diabetes Mellitus*, hypertension, and coronary artery disease and is therefore a major contributor to morbidity and mortality (Tontonoz *et al.*, 1994).

  The adipogenic differentiation is a complex process involving critical changes in cell phenotype and gene expression, in the process, a number of transcription factors have

been identified as potential regulators, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). During differentiation, cells become spherical in shape and accumulate small lipid droplets in their cytoplasms. These droplets later fuse to give the cells the rounded appearance shown by the cells of mature white adipose tissue. The mature adipocyte produced by *in vitro* differentiation has many of the characteristics of adipose cells *in vivo*. They are sensitive to metabolic effectors in a similar way to those of adipose tissue.

Terminal differentiation to adipocytes involves alteration in more than 100 genes with the time course of differentiation reflected by the appearance of early and late mRNA markers. These changes are primarily at the transcriptional level, although post-transcriptional regulation occurs for some adipocyte genes (Smas *et al.*, 1995).

3T3L1 is a cell line which has become an standard for the study of adipocyte differentiation. Conversion of 3T3-L1 pre-adipocyte cells into fat-adipocytes occurs in about 1 week following exposure of confluent cells to appropriate hormonal reagents.

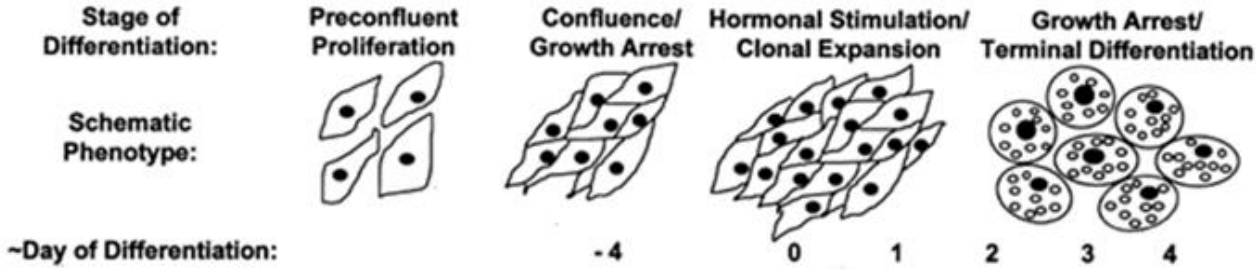


Figure 4. Summary of in Vitro adipocyte differentiation (Modified Cowherd *et al.,* 1999).

- **Osteoblast differentiation**

The osteoblast differentiation and bone formation are regulated by specific growth regulatory and transcriptional factors like the bone morphogenetic proteins (BMPs) and its downstream signalling molecules Smad 1 and Smad 5 as well as RUNX-2.

Bone formation is regulated by a number of growth regulatory factors, key signal transduction molecules and critical transcription factors. Important among these are the bone morphogenetic proteins (BMPs) which promote normal appositional bone growth and induce ectopic bone formation experimentally. The mechanism of action and

regulation of BMP signalling molecules during osteoblast differentiation are not fully defined (Zhao *et al.*, 2003).

Wnt/β-catenin pathway

β-catenin is the central effector molecule of the canonical Wnt signalling pathway, which governs cell fate and differentiation during embryogenesis. In the absence of signalling β-catenin is phophorylated/ubiquitinated through a complex of proteins, like Axin. When the Wnt ligands bind to Frizzled and LRP5/6 receptors, the pathway is activated and a signalling cascade is initiated through Dishevelled that leads to inhibition of proteosomal degradation of β-catenin. Non-phosphorylated β-catenin accumulates and translocates to the nucleus (Siapati *et al.*, 2010).

Problem, Hypothesis and Objectives

Most studies on Smurf1 are based in osteoblast precursor cells like the line 2T3. However our study is based in pre-adipocyte cells, the mouse line 3T3-L1. Our goal was to investigate the role of Smurf1 in the differentiation 3T3-L1. For this, we silenced Smurf1 in this cells line, using two different commercial lentiviruses.

Our hypotheses were:

- Silencing of Smurf1 would result in an increase in their targets like Runx-2, the BMP-1A´s receptor and BMP-2´s receptor.

- As a consequence, undifferentiated 3T3-L1 cells would differentiate to osteoblast rather than adipocyte phenotype.

- The 3T3-L1 cells with ShRNA Smurf1 would have more β-catenin in the cytoplasm and in the nucleus.

Materials and Methods

Antibodies and Reagents

Puromycin 1 µg/mL, Ampicillin 1:1000, SH573 vector, SH574 vector, PLKO1-puro, penicillin-streptomycin Polybrene 1 µg/mL Oil-Red O and GenElute Plasmid MIDIprep Kit were obtained from Sigma (Madrid, Spain), Reverse-transcription reagents including SuperScript II™ reverse transcriptase were acquired from Bio-Rad (Madrid, Spain) , IBMX 0.5 mM, Dexamethasone 1 µM, insulin 1 µM, Indomethacin 2µM Trypsin/EDTA 10x were got from Lonza (Barcelona, Spain), SYBR® Green Real-Time PCR Master Mixes and Trizol® Reagent were come by Life-technologies (Madrid, Spain). Finally the Primers to Real-Time PCR were obtained from Biolegio (Nijmegen, Netherlands).

Cell Culture

Human 293-T cells from ATCC (American Type Culture Collection, CRL-11268™), derived from human kidney were maintained in Dulbecco´s modified Eagle´s growth medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37ºC in 5 % CO_2 with humidification. Cells were seeded on a 100-mm culture dish at a cell density of $5x10^5$ and the medium was changed every 2 days, when the cells reached 90% confluence, they were trypsinized, for counting in a Neubauer chamber and re-seeded for transfection at a density of 4×10^4 per 60 mm dish

Mouse 3T3-L1 preadipocytic cells from ATCC (American Type Culture Collection, CL-173™) were maintained with growth in Dulbecco´s modified Eagle´s (DMEM) supplemented with 10% Donor Bovine Serum (DBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37ºC in 5% CO_2 with humidification. $5x10^5$ cells were seeded on each 100-mm culture dish and the medium was changed every 2 days.

All the experiments were performed with cells with 2 to 10 passes.

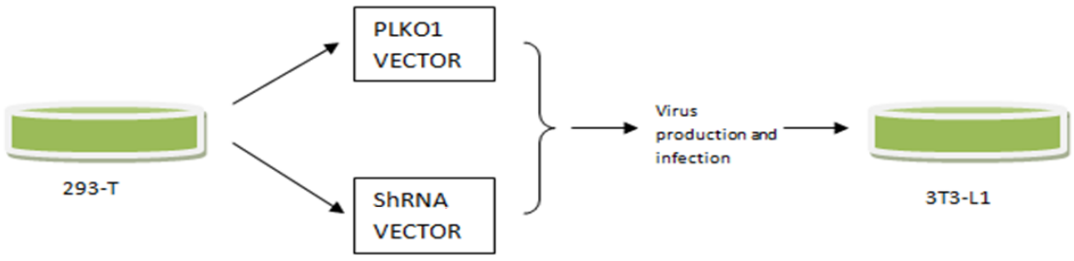
Construction of Smurf1-Lentiviral Vector and Lentiviral Infection

Two different Sh's specific for Smurf1 (Sigma Aldrich, US) cloned in the vector pLKO- Puro vector were used for Smurf1 silencing. The sequences of the sh's were SH573: 5´-CCGGCCAGTATTCCACGGACAATATCTCGAGATATTGTCCGTGGAATACTGGTTTTTG-3´ and SH574: 5´-CCGGGCCAAGAACCTTGCAAAGAAACTCGAGTTTCTTTGCAAGGTTCTTGGCTTTTTG-3´.

DNAs for lentiviral production were prepared with Qiagen MiniPrep from cultures started from single colonies obtained from glycerol stocks. Both SH clones, the empty vector pLKO1-puro, and the packaging plasmids VSVG and psPAX2 were grown overnight in 100 mL LB Broth-ampicillin with vigorous shaking, and then prepared according to the manufacturer's protocol.

Lentiviral stocks were produced by transient transfection of 293-T cells with recombinant plasmids by the calcium phosphate precipitation method (Graham and Van der Eb 1973). Briefly, 4 x 10^4 cells were plated on a 100-mm culture dish with growth medium. After 24 hours the transfected with 20 µg of transfer vector (our SHRNA and PLKO1-empty), 15 µg of the packaging plasmid psPAX2 and 6 µg of the envelope plasmid pCMV-VSVG. The medium was replaced with 6.5 mL of warmed medium after 9-14 hours. 2 days later the viral particles were collected by centrifugation of the medium. Several rounds of viral particles collection were carried out at different days to enrich the viral count. Finally, all supernatants were filter through a 0.45 µm filters (Milipore, Belford, MA) collected by centrifugation, aliquoted and stored at -80ºC until use.

For lentiviral infection, 15000 3T3-L1 cells were seeded on a 60 mm culture dish. Cells at 40% confluence were infected by adding 30 µL of lentiviral and polybrene 4 µg/mL. After 48 hours, the medium was changed and 1.5 µg/mL of Puromycin were added to select the infected cells. Cells were further cultured for 2 weeks and green fluorescent colonies were picked and seeded in 24-well culture plates and, when grown, changed to 100-mm culture dishes. In this process, the medium was changed every 2 days and Puromycin selection was maintained. Periodical observations at the fluorescent microscope were done to verify that they expressed the GFP (Green Factor Protein) meaning that the virus had not been lost.



Cell Growth Assay

Cells were grown at a density of 30000 per well. At 24- hours intervals (24, 48 and 78hrs respectively) the cells were trypsinized and counted in a hematocytometer or in a Neubauer chamber.

Cell Differentiation Induction Assay and Oil-Red Staining

A standard protocol was employed for adipocyte differentiation. First, cells on 24-well plates at a cell density of 10000 cells per well. Two controls, 3T3-L1 without any treatment and 3T3-L1 infected with PLKO1-puro and cells silenced fro Smurf1 with two different viruses, SH573 and SH574, were seeded by duplicate. The differentiation was induced when the cells reached 80% confluence by replacing growth medium with DMEM containing 10% Donor bovine serum (DBS), 1 µM of insulin, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and Indomethacin 2µM.The medium was changed at 2-day intervals Washing with PBS was avoided to prevent cells detachment. The days after the induction, the cells adopted an characteristic appearance with triglyceride droplets in the cytoplasma. Every 3 days, pictures were taken every 3 days to follow the changes. Also the cells were fixed with 4% formaldehyde for 30 minutes a room temperature, and washed twice with PBS 1X. Oil-Red (prepared by diluting a stock solution containing 0.5 g of Oil Red O per 100 mL of isopropanol with water (6:4) followed by filtration). The Oil-Red O was left for 15 minutes and washed it 3-4 times with PBS 1X. The last day, 9 days after the induction plates were sealed and analyzed by microscopy.

Inmunofluorescence Study

Cells were seeded on small slides at 30000 cells per dish in 24-well plates. At 80 % confluence, cells were fixed with 3.7% formaldehyde during 15 minutes at room temperature. Then, the cells were washed with PBS 1X three times during 5 minutes. During 30 minutes the cells were permeabilized with PBS 1x 0, 5% Triton X-100 and 0,1M Glycine, and again washed three times for 5 minutes with PBS 1X and then they were washed with PBS 1X + 0.05% Tween20 for 2 minutes (the Tween20 is a detergent solution that decreases the surface tension). After this, the primary antibody diluted in PBS 1X + 0.05% Tween20 + 3%FBS solution was added and incubated "overnight" in a wet chamber at 4ºC. The next day, after three 5-minute washes with PBS 1X and another wash with PBS 1X + 0.05% Tween20 during 2 minutes, the secondary antibody diluted (1:500) in PBS 1X + 0.05% Tween20 + 3% FBS was added and incubated for an1 hour.

Finally, one drop of DAPI was added on the slide, and the slide was put facedown the dish with the cells and let dry "overnight" at room temp and in darkness. Sealed it with nail polish and looked at fluorescence microscope.

RNA Extraction and Reverse Transcription

The different 3T3-L1 treatments were seeded in a 24-well plate at 30000 cells per well and grown until 80% confluence. Then they were trypsinized and lysed for 5 minutes with 500 µL of TRIZOL®. Then100 µL of chloroform were added for 15 seconds, followed by an incubation of 3/5 minutes at room temperature and an spin at 12000 rpm during 15 minutes at 4ºC. Then, the upper phase was collected on 250 µL of isopropanol left for 10 minutes at room temp, spun during 20 minutes at 4ºC and the pellet was washed with 500 µL of ETOH 75%. The dried pellet was suspended in RNase-free water and kept at -135ºC until use.

The concentration of the isolated RNA was measured in a nanodrop. Reverse transcription to synthesize cDNA was carried out in a 20 µL volume with 0.5 µg of total RNA, 4 µL 5x iScript reaction mix, 0.8 µL iScript reverse transcriptase and x µL Nuclease-free water (Bio-Rad Madrid, Spain). The mix was incubated during 5 minutes at room temperature, 30 minutes at 42ºC, 5 minutes at 85ºC and 5 minutes at 4ºC.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed to determinate the mRNA expression level of Smurf1, Fabp4, adiponectin, osteocalcin, osteogenin, BMPR-1A, BMPR-2, RUNX-2 and Axin-2.

The sequences of the primers were as follows:

Gene	**Sequence**		**Size (bp)**
	Forward	**Reverse**	
Smurf1	5´-TGCCATCAGCAGATTGAAAG-3´	5´-GTTCCTTCGTTCTCCAGCAG-3´	174
Fabp-4	5´-TCACCTGGAAGACAGCTCCT-3´	5´-ÁATCCCCATTTACGCTGATG-3´	182
Adiponectin	5´-GTTGCAAGCTCTCCTGTTCC-3´	5´-TCTCCAGGAGTGCCATCTCT-3´	192
Osteocalcin	5´-GCGCTCTGTCTCTCTGACCT-3´	5´-ACCACTCCAGCACAACTCCT-3´	175

Osteogenin	5´-TGCTGTGGCTCTATGACAGG-3´	5´-ACCCCTTCGTTTGAGGAGTT-3´	153
BMPR-1A	5´-AGGTCAAAGCTGTTCGGAGA-3´	5´-CTGTACACGGCCCTTTGAAT-3´	178
BMPR-2	5´-TGGCAGTGAGGTCACTCAAG-3´	5´-TTGCGTTCATTCTGCATAGC-3´	178
Runx-2	5´CCCAGCCACCTTTACCTACA-3´	5´-TATGGAGTGCTGCTGGTCTG-3´	150
Axin-2	5´-CTCCCCACCTTGAATGAAGA-3´	5´-ACTGGGTCGCTTCTCTTGAA-3´	167

Thirteen microliters of reaction volume included 1 µl of cDNA, 0.26 µl of 10 µM of each primer, 6.5 µl of 2 x SYBR green master mix and 4.98 µl of water MiliQ. Real-time PCR was performed with a specific protocol: 40 cycles at 57ºC. To confirm the amplification specificity, the PCR products were subjected to a dissociation curve analysis. The threshold cycles (Ct) of each reaction were normalized to those 18S mRNA using the $^{-\Delta\Delta}$Ct method. All PCR reactions were performed in duplicate.

Data Analysis

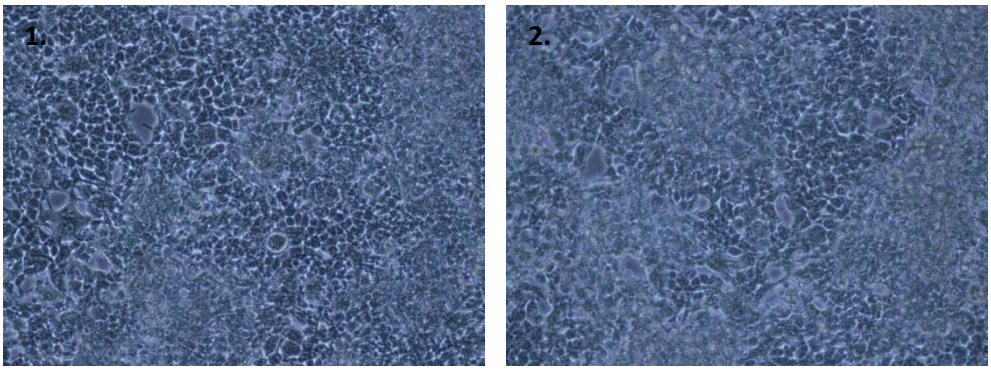
Results are presented as the mean of two or three determinations (n) without error bars; because all experiments are in this report.

Results and Discussion

Smurf1 Silencing with Sh-lentiviral Vector

With the purpose of generate Smurf1-silencing in the 3T3-L1 cells, we used two commercial lentiviral plasmids, SH573 and SH574 and the empty vector (PLKO1) as a negative control. Lentiviruses for either sh or the control were produced in 293-T cultures, as described in the material and methods section. The green fluorescence indicates the presence of cells expressing the GFP encoded by the plasmid (Fig.5).

A)



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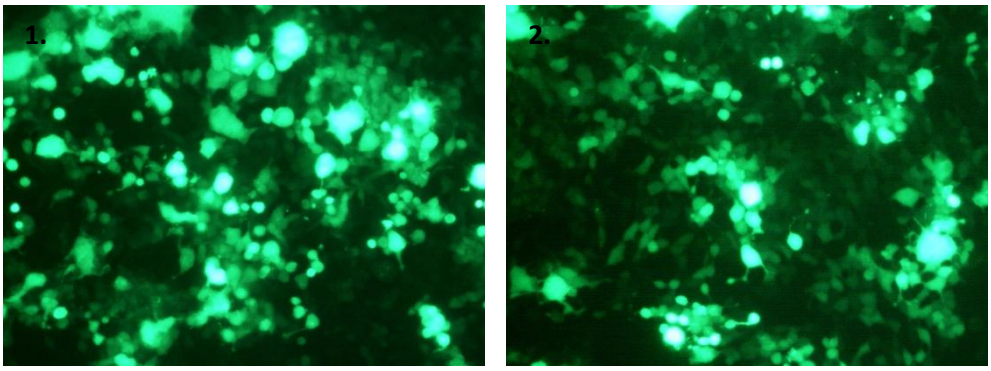


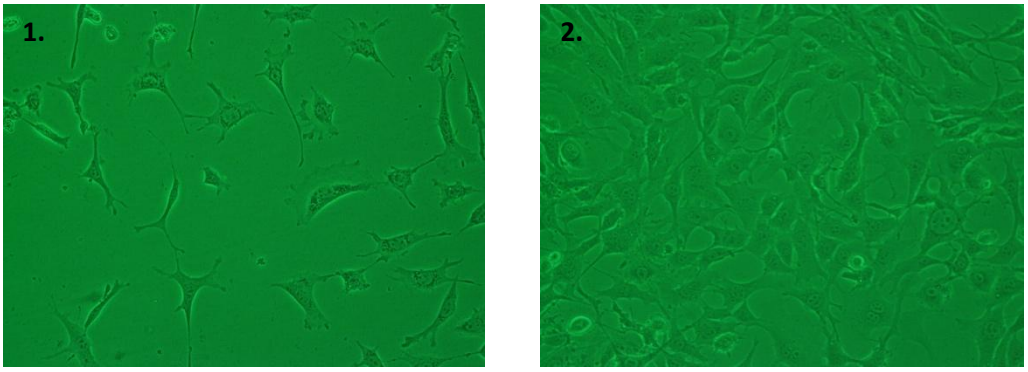
Figure 5. Lentiviral´s production in 293-T cells. A) 1. Lentiviral Sh573 vector production without fluorescence. 2. Lentiviral Sh574 vector production without fluorescence. B) 1. Lentiviral Sh573 vector production with fluorescence. 2. Lentiviral Sh574 vector production with fluorescence.

Once the lentiviral suspensions were prepared, they were added to 3T3-L1 cultures. Infected cells were selected with the antibiotic Puromycin (1µg/ml). Puromycin- resistant cells which also expressed GFP (Green Fluorescent Protein) were considered to have been Smurf1 silenced (Fig.6). Several clones were isolated, grown for and the levels of Smurf1 mRNA were tested by

RT-PCR (Fig.7). According to our observations Smurf1 silencing is partially lost with time and often the low expression of Smurf1 seen in the first passes is increased in the last passes (Fig3).

Also our observations indicated that Sh574 viruses were more efficient in silencing Smurf1 expression in the 3T3-L1 than their Sh573 counterparts.

A)



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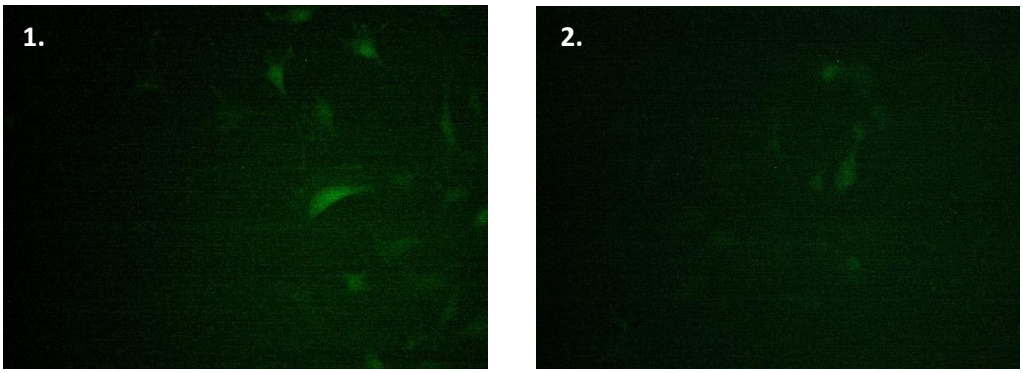


Figure 6. Infection in 3T3-L1 cells with the different lentivirals. A) 1. 3T3-L1 cells with Sh573 vector without fluorescence. 2. 3T3-L1 cells with Sh574 vector without fluorescence. B) 1. 3T3-L1 cells with Sh573 vector with fluorescence. 2. 3T3-L1 cells with Sh574 vector with fluorescence.

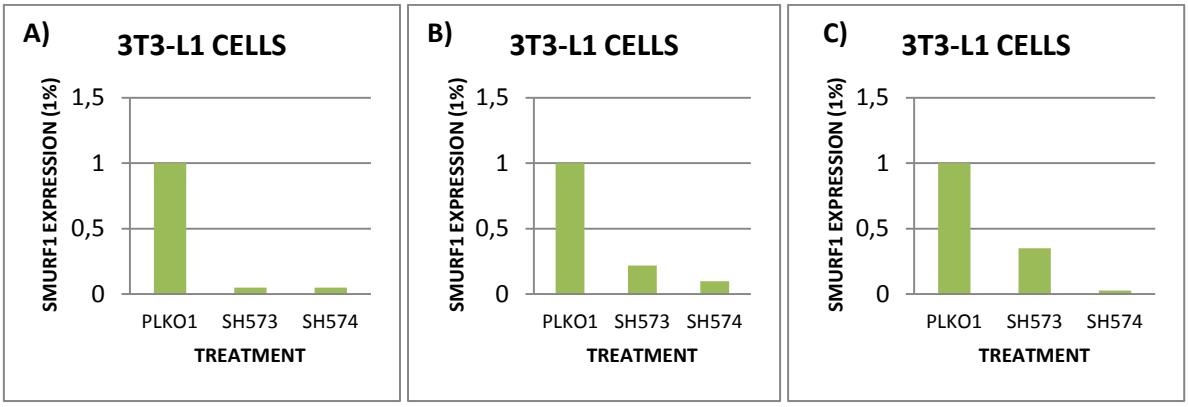


Figure 7. RT-PCR Smurf1 expression. Smurf1 silencing in 3T3-L1 cells with different passes. A) 3T3-L1 cells with Smurf1 silencing in the 2nd pass. B) 3T3-L1 cells with Smurf1 silencing in the 4th pass. C) 3T3-L1 cells with Smurf silencing in the 6th pass.

Smurf1 Silencing slows the 3T3-L1 proliferation

Smurf1- silenced cells proliferate significantly less than the control cells. On average, 573 clones showed a higher proliferation rate than 574 clones which was in accordance with the differences in Smurf1 silencing observed previously (Fig. 8).

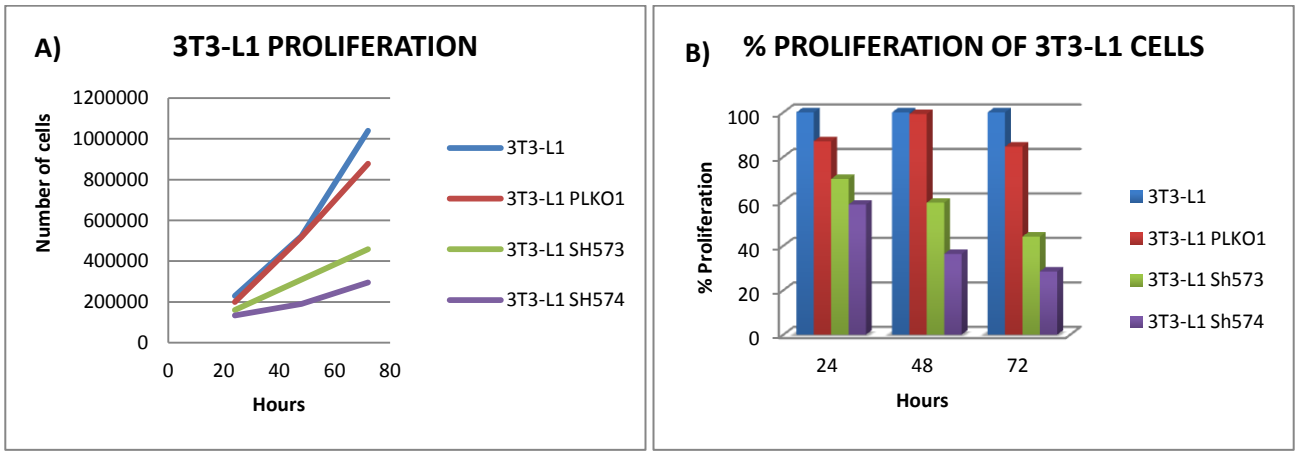


Figure 8. Cells Growth Assay. A) N=3. Average cell proliferation during 3 weeks. The number of cells was counted every 24 hours during 72 hours and repeats 3 times. B) Percent of proliferation for each treatment.

The effect of Smurf1 silencing in the β-catenin pathway

As Smurf1 participates in the degradation of proteins of the canonical/Wnt pathway, we hypothesized that silencing of Smurf 1 would result in an activation of the pathway. To test this hypothesis we analyzed whether there is an increase in β-catenin in the cytoplasm and nucleus in the 3T3-L1. Immunofluorescence assays showed an increase in the amounts of β-catenin in 3T3-L1 silenced with Sh574 which suggests an activation of Wnt canonical pathway (Fig. 9).

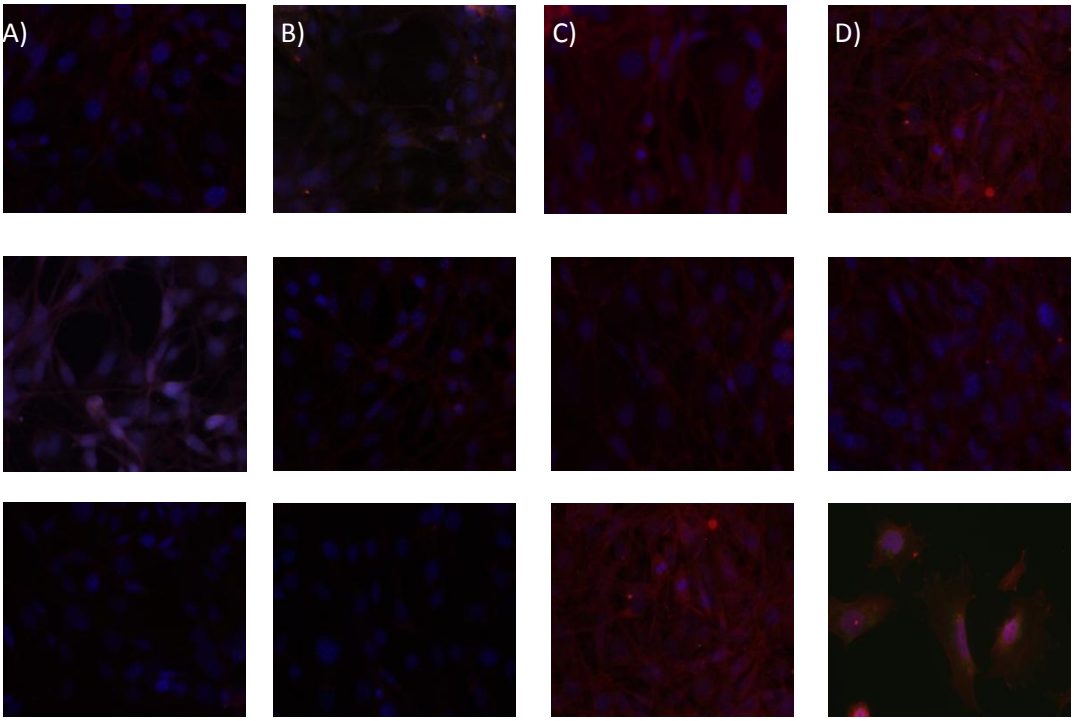


Figure 9. Inmunofluorescence Assay with cells taken in different days (Different passes). A) 3T3-L1 cells without any treatment. B) 3T3-L1 cells with PLKO1 vector. C) 3T3-L1 cells with Sh573 vector. D) 3T3-L1 cells with Sh574 vector, the last photo used the objective X40.

To further investigate this, we analyzed Axin-2 (a protein that plays an important role in the regulation of the stability of the β-catenin) expression by RT-PCR. Our results also showed an increase in axin-2 gene expression after silencing (Fig. 10).

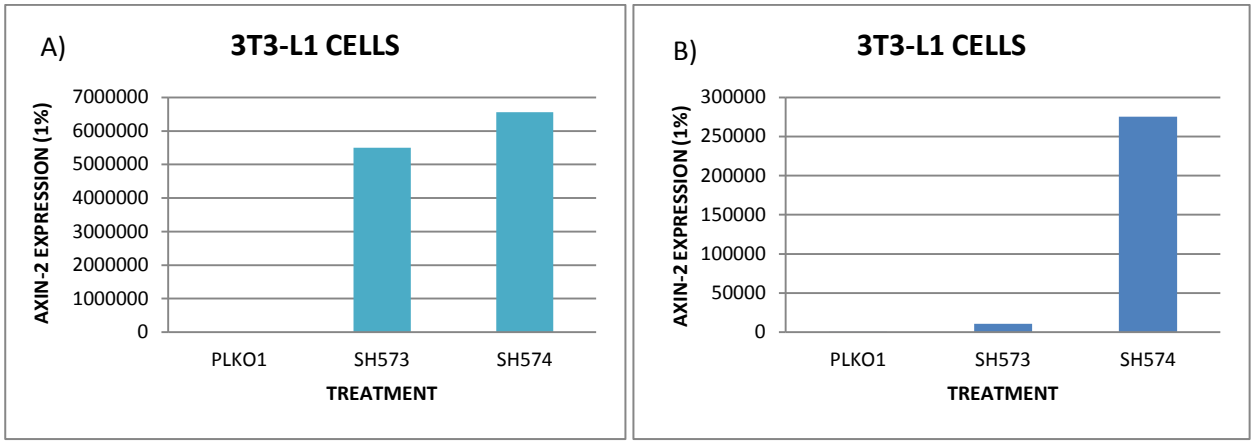


Figure 10. RT-PCR **Axin-2 expression.** A) Axin-2 expression in 3T3-L1 cells with PLKO1 vector, Sh573 vector and Sh574 vector in 2nd pass. B) Axin-2 expression in 3T3-L1 cells with PLKO1 vector, Sh573 vector and Sh574 vector in 4th pass.

Then the RT-PCR products run on an Agarose 1% gel through electrophoresis (Fig.11).

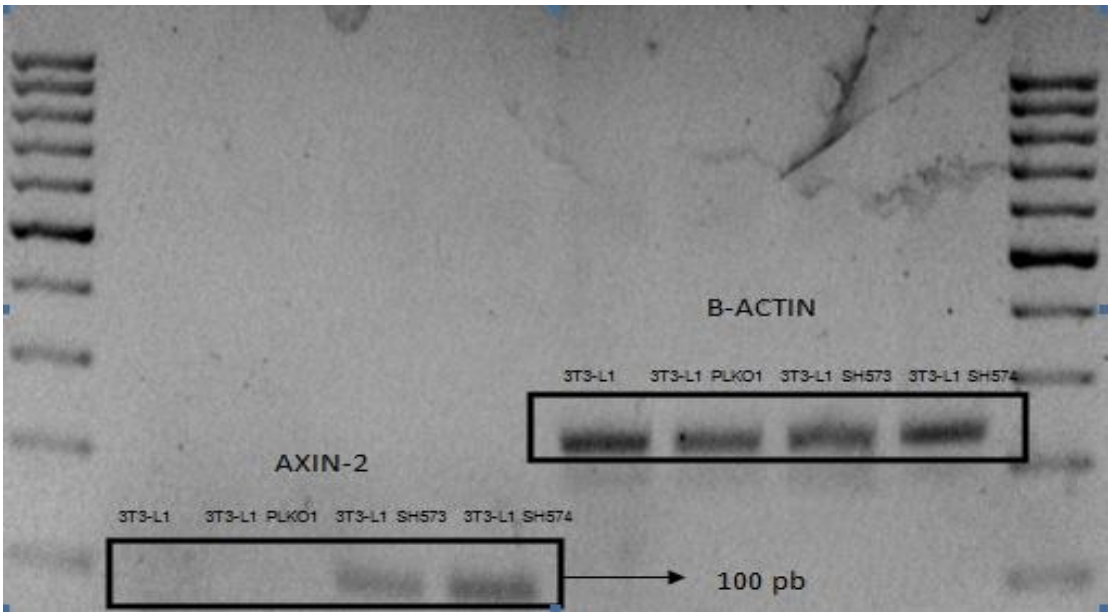


Figure 11. Gel electrophoresis (Agarose 1%). There is amplification in the 3T3-L1 cells with Smurf1 silencing.

Smurf1 Silencing causes a blockage in the adipogenic differentiation of 3T3-L1

Control 3T3L1 cells can go through a complete adipogenic differentiation process. This was not observed in Smurf1- silenced 3T3-L1 cells, which show an aberrant differentiation. Because Smurf1 might participate in osteoblast differentiation, we also analyzed the possibility that the silenced cells undergo a osteoblast differentiation. This was achieved by several different

methods, like morphological analysis, Oil-red staining (Fig. 12, 13, 14, 15) and analysis of the expression of differentiation markers by RT-PCR.

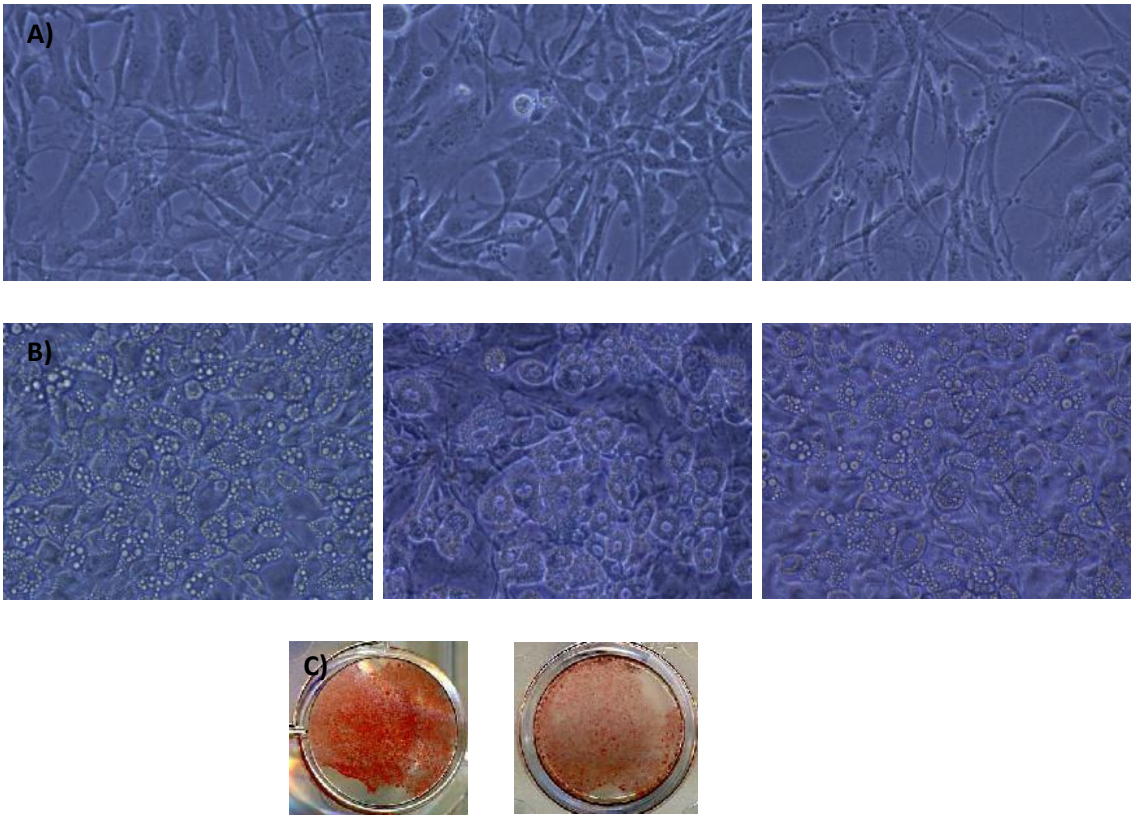
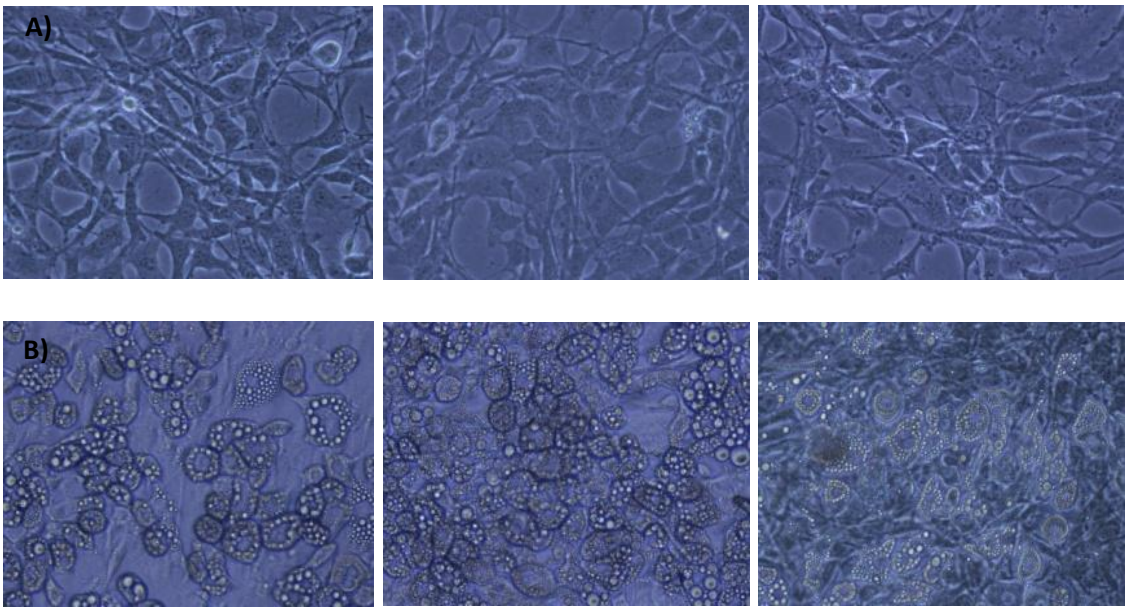


Figure 12. 3T3-L1 cells without any treatment, different passes. A) Non-differentiate cells. B) After 9 days differentiate. C) After 9 days differentiate and stain with Oil-Red.



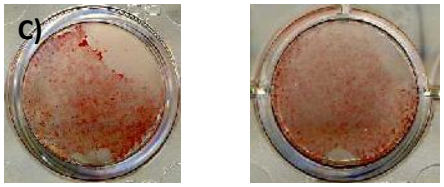


Figure 13. 3T3-L1 cells with PLKO1 vector, different passes. A) Non-differentiate cells. B) After 9 days differentiate. C) After 9 days differentiate and stain with Oil-Red.

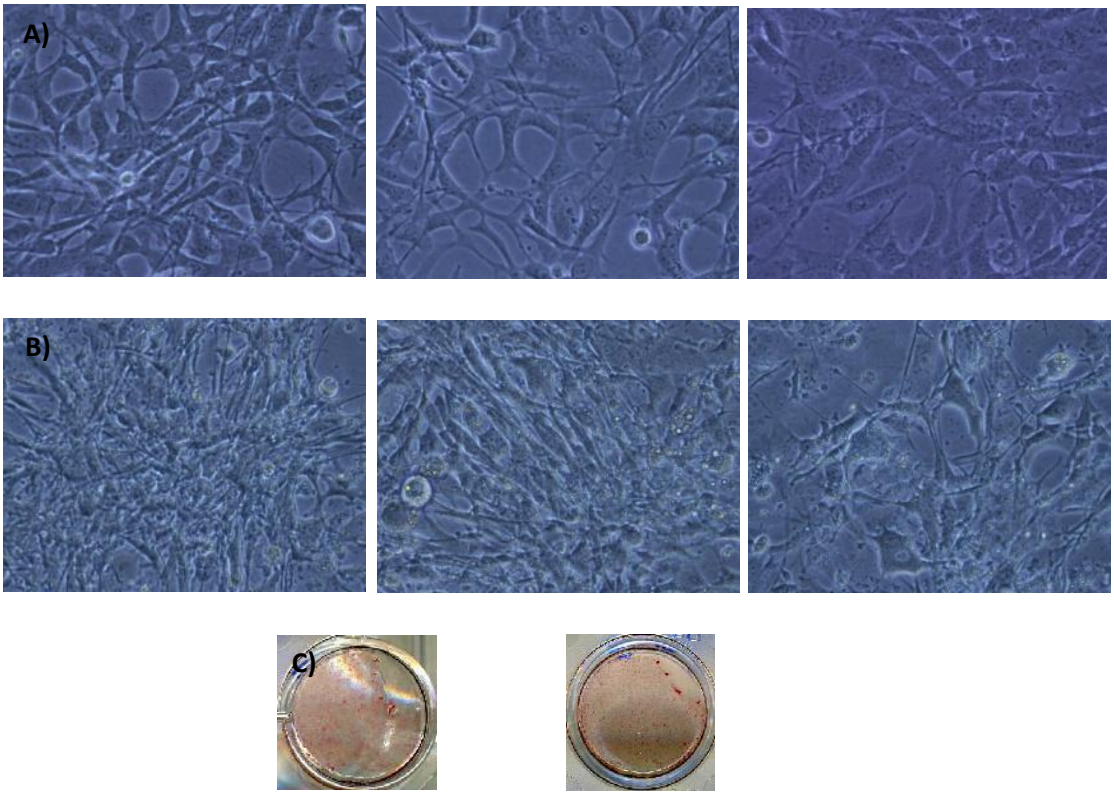
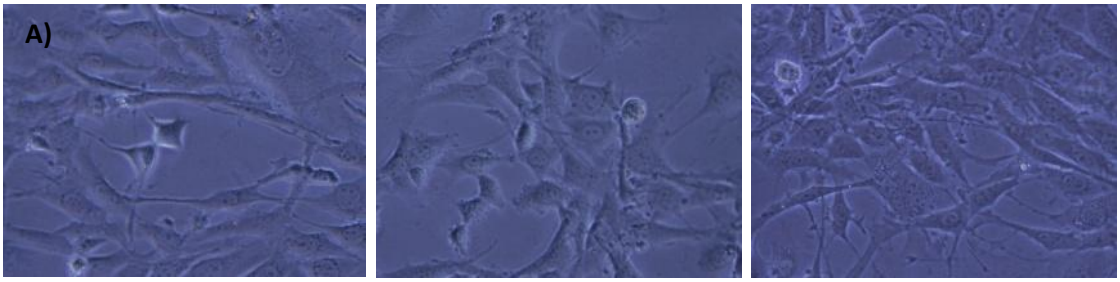


Figure 14. 3T3-L1 cells with SH573 vector, different passes. A) Non-differentiate cells. B) After 9 days differentiate. C) After 9 days differentiate and stain with Oil-Red.



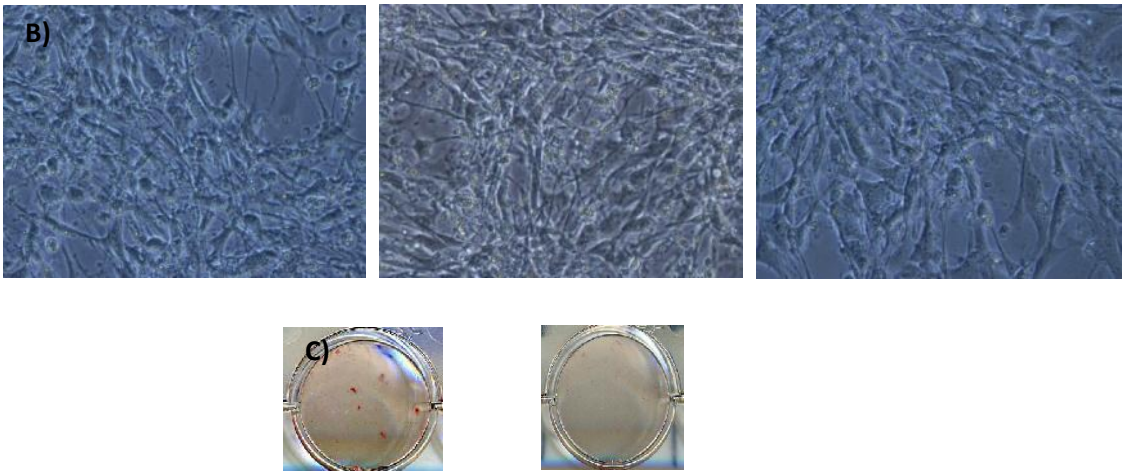
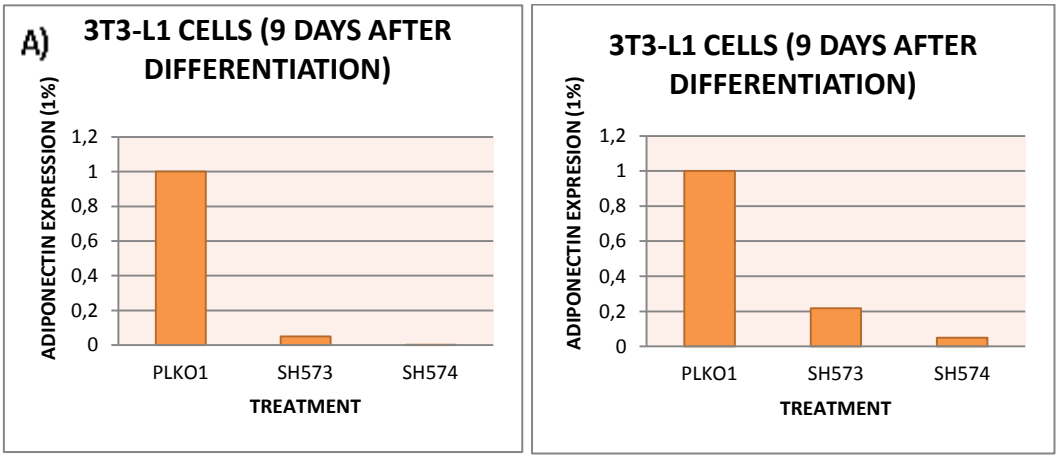


Figure 15. 3T3-L1 cells with SH574 vector, different passes. A) Non-differentiate cells. B) After 9 days differentiate. C) After 9 days differentiate and stain with Oil-Red.

Three different markers were used: the adipogenic markers Adiponectin and Fabp4 and the osteoblast marker: Osteocalcin. The results indicated that Smurf silencing (also checked during the analysis) blocks adipocyte differentiation. Unfortunately we weren't able to get a good amplification of the osteocalcin gene and we were not able to see whether the cells were directed to an osteoblast phenotype (Fig. 16).



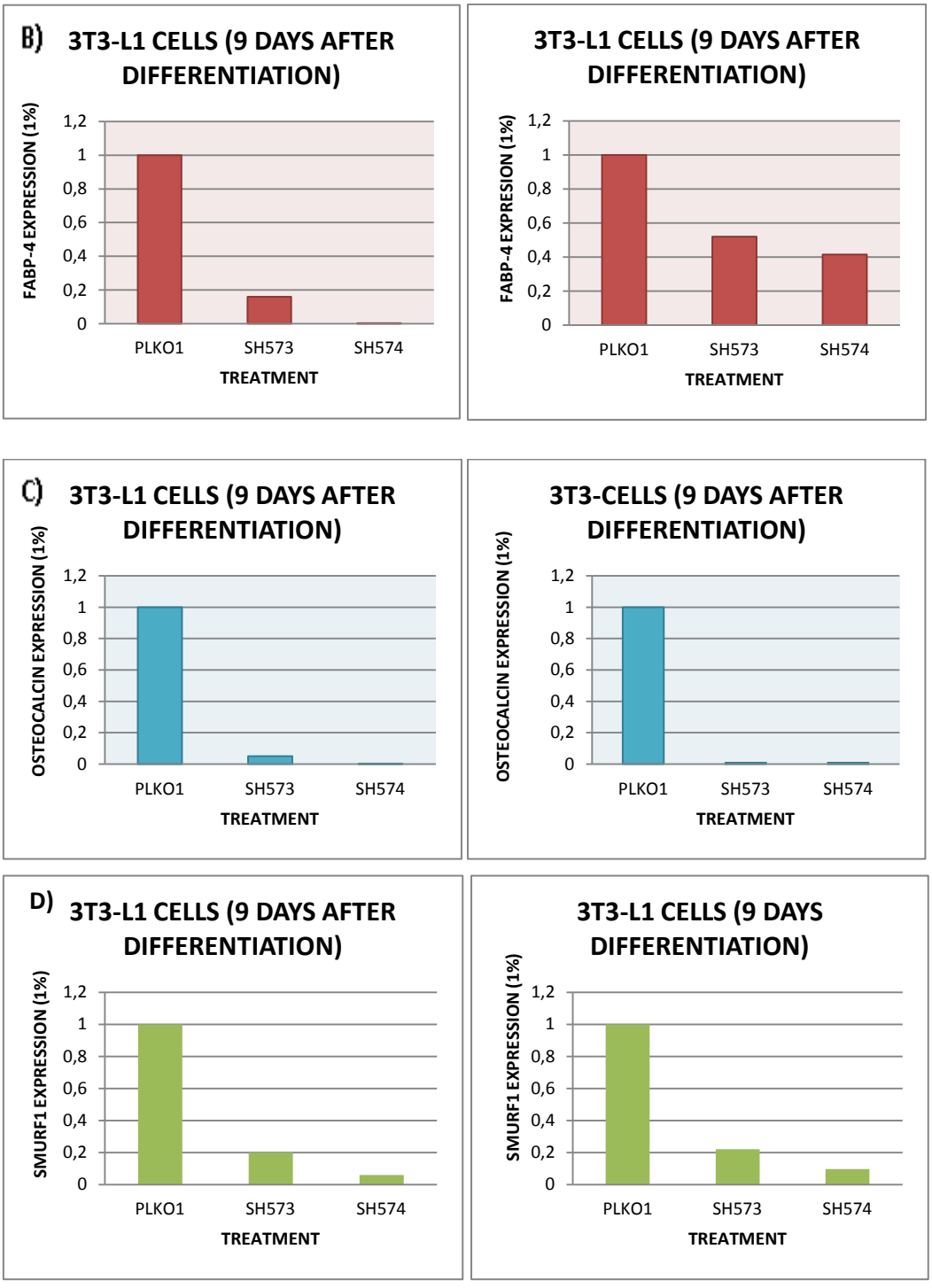


Figure 16. RT-PCR, 3T3-L1 cells 9 days after differentiation. A) Adiponectin expression. B) Fabp4 expression. C) Osteocalcin expression. D) Smurf1 expression.

Smurf1 Silencing does not affect the mRNA levels of Smurf1 targets

The mechanism of action of Smurf1 consists in preparing its targets for degradation. Therefore it was not very likely to see changes in the levels of mRNA of its targets. To confirm that there was not any transcriptional effect interfering with the effect of Smurf silencing we measured the expression Runx-2, BMPR-1A and BMP-2 (Fig. 17).

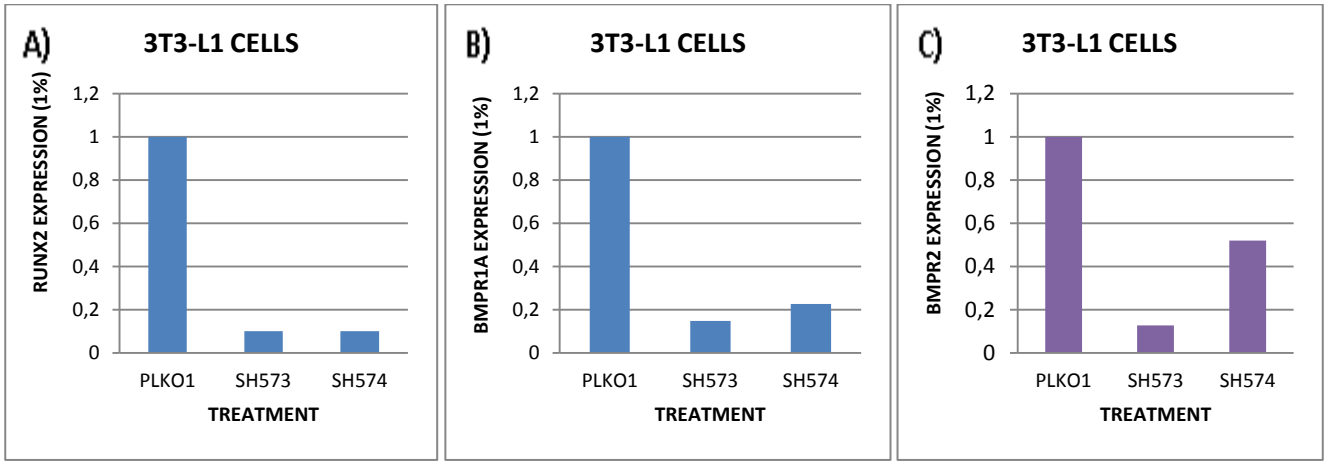


Figure 13. RT-PCR Smurf1 targets. A) Runx-2 expression. B) BMPR-1A expression. C) BMPR-2 expression.

Conclusions and Significance

Throughout of this project, we have drawn several conclusions that can have a great importance in further research.

- Smurf1 silencing in 3T3-L1 cells show a lower proliferation rate. This might be due to the presence of higher levels of Smad1, a Smurf1 target, which participates in both TGF-β and BMPs pathways.

- In 3T3L1 Smurf1 silencing blocks adipogenic differentiation. Smurf1 silencing activates the canonical Wnt/β-catenin pathway, which is characteristic of bone differentiation. At this moment we are not able to determine if cells undergo an osteoblast differentiation but studies are under way.

This project emphasizes the role of Smad Ubiquitin Regulatory Factor-1: Smurf1 in the adipogenic differentiation.

Applications and Further Research

Our work gives us the opportunity to open a range of possibilities that relate Smurf1 with different pathologies such as the insulin resistance (*Diabetes Mellitus*) and the obesity.

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