The role of the Smad Ubiquitin Regulatory Factor-1: SMURF1 in the differentiation 3T3-L1

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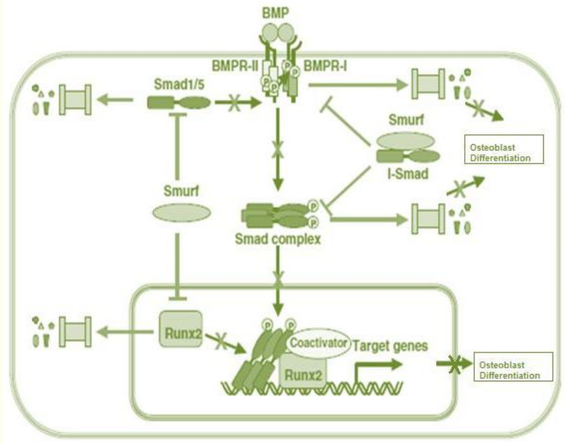
INTRODUCTION AND PRIOR RESEARCH

The Smad Ubiquitin Regulatory Factor-1: SMURF1 is an E3 ligase. This E3 ligase has been linked with 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 (1). Previous studies, in 2003, have demonstrated that overexpression of Smurf1 induces proteasomal degradation of Smad1 and Runx2 proteins by the mechanism shown in the figure (2,3). Runx-2 is a transcription factor that is essential for osteoblastic differentiation, bone formation and maintenance, Both the pre-adipocyte cells and osteoblast cells are originated from common mesenchymal progenitor cells and it has been recently demonstrated that Runx2 is also involved in adipocyte differentiation(4).



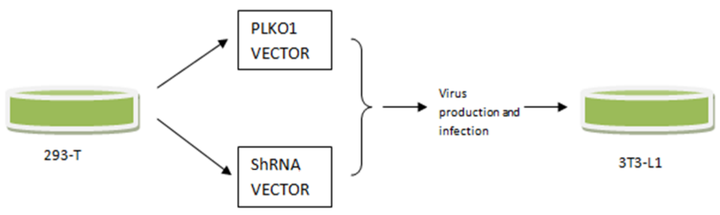
PROBLEM AND HYPOTHESIS

Most studies onSmurf1 are based in osteoblast precursor cells. However our study are based in pre-adipocyte cells, 3T3-L1. Our goal was to investigate the role of Smurf1 in the differentiation 3T3-L1. For this, we silenced Smurf1 in this cell line, using two different commercial lentiviral´s vector (shRNA).
Our hypotheses were:
a) The cells with ShRNA Smurf1 would proliferate less than the control´s cells.
b) The cells with ShRNA Smurf1 would be differentiated to osteoblast phenotype.
c) The cells with ShRNA Smurf1 would be increased their target like RUNX-2 and BMP´s Receptors.
d) The cells with ShRNA Smurf1 would have more β-catenin in the cytoplasm and in the nucleus.

MATERIALS AND METHODS

A) Construction of Smurf1-Lentiviral Vector and Lentiviral Infection

-2 ShRNA to silence Smurf1 → Sh573 and Sh574
-PLKO1 vector → negative control
-Packing cell line→293-T cells (DMEN +10%FBS)



B) Cell Growth Assay

-Count each cellular treatment every 24 hours by the Neubauer chamber during 72 hours →Proliferation

C) Cell Differentiation Induction Assay and Oil-Red Staining

-In each treatment were added to the DMEM 10%DBS:
1µM of Insulin
1µM of dexamethasone
0.5mM 3-isobutyl-1-methylxanthine
2µM of Indomethacine.
Keep the cells during 9 days, fix and stain the different cellular treatments with the Oil-Red

D) Inmunofluorescence Study

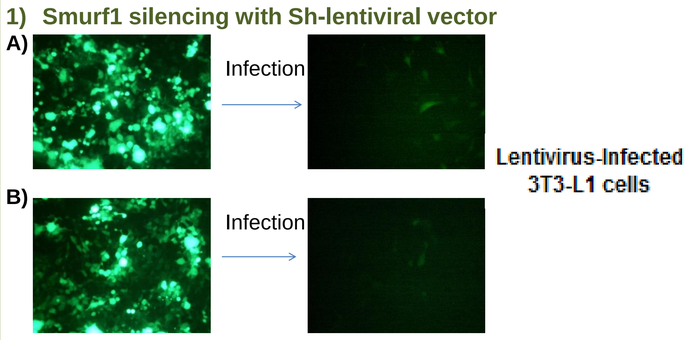
-Fix the cells when reach 80% confluence and permeabilize then
-Add and incubate the cells with the primary antibody “overnight” in a wet chamber
-Add and incubate the cells with the secondary antibody and add DAPI to stain cells

E) RT-PCR

-RNA extraction for each treatment, both differentiated cells as undifferentiated cells → Reverse transcription → 0.5 µg cDNA
-mRNA expression level to:
Smurf1
Axin-2
Smurf1 targets: Runx-2, BMPR-1ª and BMPR-2
Adipogenetic differentiation: Adiponectin and FABP-4
Osteoblastic differentiation: Osteocalcin.

RESULTS AND DISCUSSION

1) Smurf1 silencing with Sh-lentiviral vector



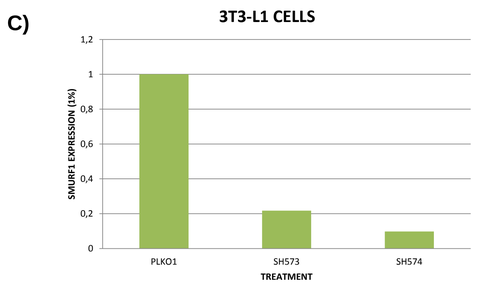


Figure 1. Smurf1 silencing**.** A) Lentiviral Sh573 vector production in 293-T cells and its infection in 3T3-L1 line.
B) Lentiviral Sh574 vector production in 293-T cells and its infection in 3T3-L1 line. C)RT-PCR Smurf1 expression. Several 3T3-L1 clones were isolated by antibiotic selection.

2)Smurf1 silencing slows the 3T3-L1 proliferation

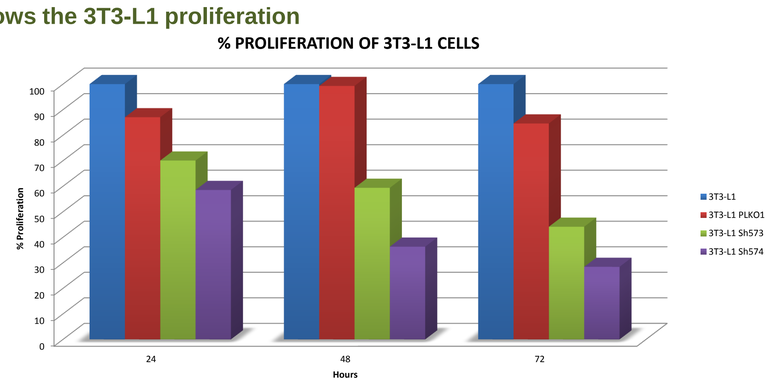
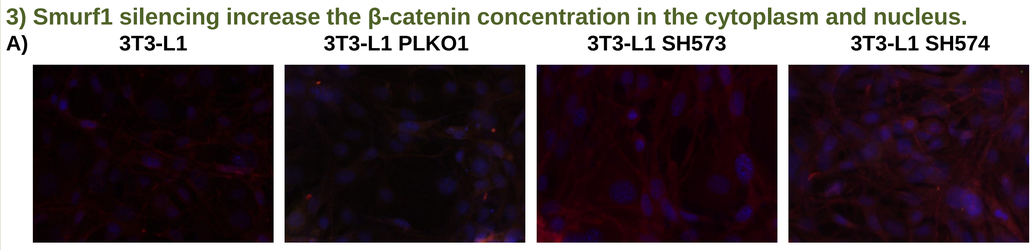
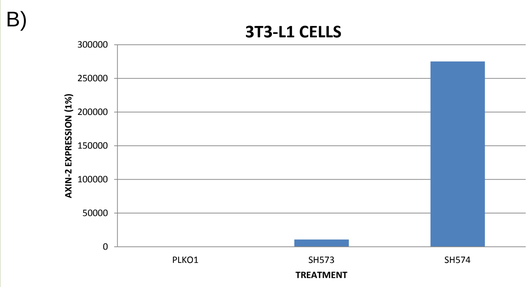


Figure 2. Percent of proliferation for each treatment, the cells were counted each 24 hours during 72 hours. 3T3-L1 cells and 3T3-L1 PLKO1 cells were our negative controls. We can see less proliferation in 3T3-L1 ShRNA compared with the controls.

3) Smurf1 silencing increase the β-catenin concentration in the cytoplasm and nucleus.





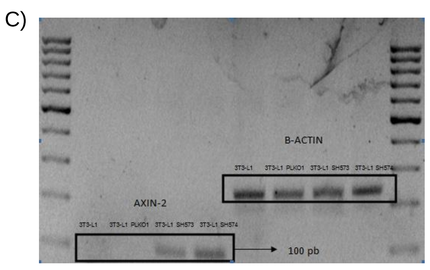


Figure 3. β-Catenin expression by different methods. A) Inmunofluorescense assay. B) RT-PCR, Axin-2 expression. C) Agarose 1% Gel Electrophoresis with RT-PCR product. There are more β-Catenin concentration in 3T3-L1 ShRNA, thus increasing the Axin-2 expression**.**

4)Smurf1 silencing causes a blockage in the adipogenic differentiation of 3T3-L1 cells

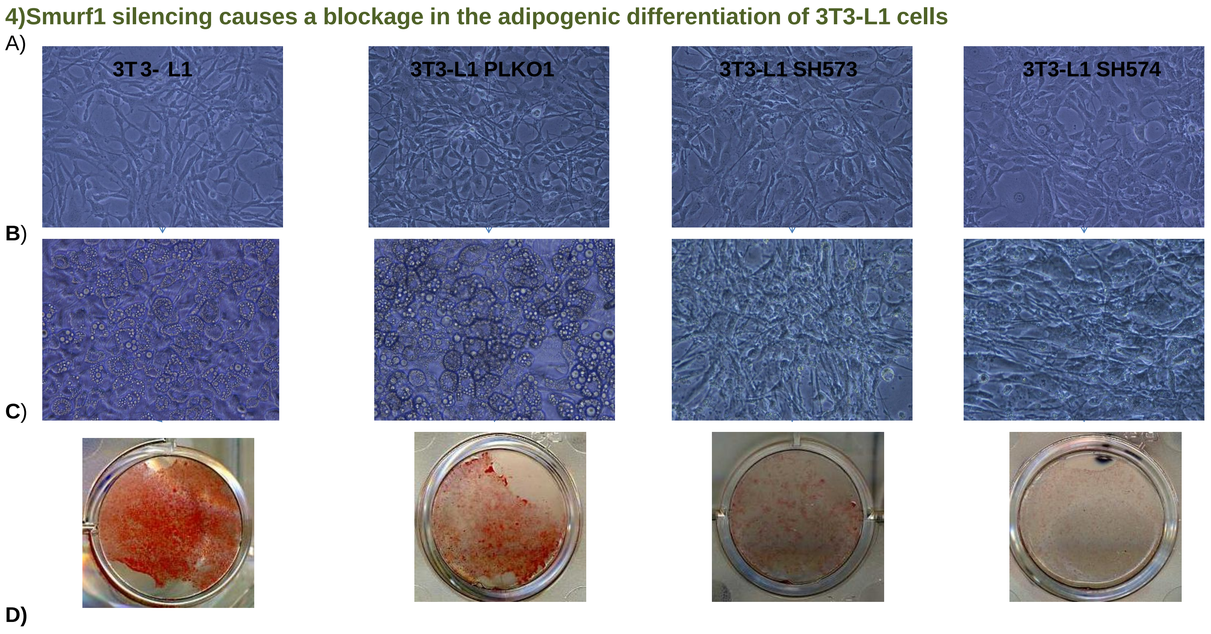


Figure 4. 3T3-L1 cells and the different treatments before differentiation and after 9 days differentiate. A) 3T3-L1 cells without any treatment, 3T3-L1 PLKO1, 3T3-L1 Sh573 and 3T3-L1 Sh574 before differentiation. B) 3T3-L1 cells without any treatment, 3T3-L1 PLKO1, 3T3-L1 Sh573 and 3T3-L1 Sh574 after 9 days differentiate. C) 3T3-L1 cells without any treatment, 3T3-L1 PLKO1, 3T3-L1 Sh573 and 3T3-L1 Sh574 after 9 days differentiate and stain with Oil-Red. D)Changes in adipocyte markers after silencing; RT-PCR Adiponectin, Fabp-4 and Osteocalcin expression.

5) Smurf1 silencing does not affect the mRNA levels of Smurf1 targets

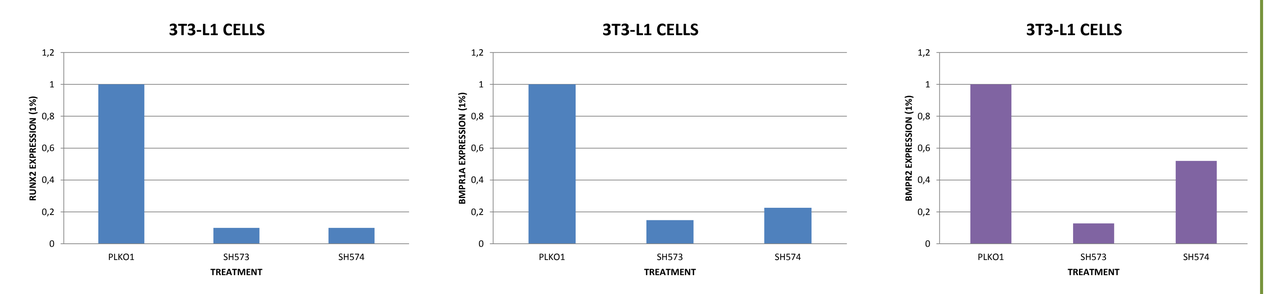


Figure 5. RT.PCR, Smurf1 targets. A) Runx-2 expression. B)BMP-1A receptor expression. C) BMP-2 receptor expression. We can not see any differences in Smurf1 targets expression at mRNA level.

CONCLUSIONS

1. Silencing of Smurf1 in 3T3-L1 cells results in a decrease in cell proliferation.

2. Adipogenic differentiation of 3T3-L1 cells is blocked after Smurf1 silencing.

3. Smurf1 silencing activates canonical Wnt/β-catenin pathway, characteristic of bone differentiation. However we weren’t able to see increases in bone markers mRNA levels in the silencing cells.

SIGNIFICANCE AND FURTHER RESEARCH

-Significance

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

-Further research

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

ACKNOWLEDGEMENTS

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