CLOSING SCIENTIFIC REPORTS

FK 155042

Principal investigator: Dr Beata Lontay

Smoothelin like protein 1 (SMTNL1) protein has dual function. In the cytoplasm it binds to myosin phosphatase (MP) and its inhibitory interaction regulate the phosphorylation state of cytoskeletal elements. On the other hand, upon PKA/PKA phosphorylation it dissociates from MP and translocates to the nucleas and acts as a progesterone receptor specific coregulator. In the absence of SMTNL1 the skeletal muscle (SKM) transforms to a glycolytic phenotype and it results in a drastic change in global metabolic properties leading to insulin resistance. Moreover, it smtnl1 KO animals show a reproductive phenotype with fertility problems. We aimed to characterize the molecular mechanism of the action of SMTNL1 in SKM as well as in endometrium. We hypothesized that SMTNL1 as a PR-B selective corespressor can downregulate the progesterone-induced shift towards insulin resistance and can also induce insulin sensitivity improving on reproductive disorders such as gestational diabetes and hyperthyroidism related insulin resistance. The translational aspect of the study was to design a PR-B binding membrane permeable peptide mimicking the specific corepressive effect of SMTNL1. The SMTNL1-mimicking peptide resulted in increased overall insulin sensitivity and a potential improvement on fertility disorders.

Work package 1: Developing progesterone receptor B (PR-B)-binding peptides to mimick the coregulatory effect of smoothelin like protein-1 (SMTNL1)

We aim to target PR-B by modulating its transcriptional activity applying cell permeable fragments of SMTNL1. We have described that SMTNL1 has exquisite selectivity to PR-B and this is the only PR-B-specific corepressor by far. SMTNL1 binds to PR-B isoform and it's speculated that an inhibitory complex is formed that is either unable to bind DNA or is incapable of binding to the transcription machinery. To promote the effect of SMTNL1 on gene expression we designed PR-B-specific interacting peptides mimicking the coregulatory effect of SMTNL1 on PR-B. We found that an N-terminal SMTNL1 peptide is necessary for PR-B binding and it increases drastically the binding capacity when compared with the full-length protein. Based on this, we designed membrane-permeable SMTNL1-mimic peptides with high affinity for binding to PR-B and containing a TAT sequence at the N-terminal end, which is required for membrane

permeability, and a biotin tag at the C-terminal end for labelling and detection, using the TAT-biotin sequence as a control. Penetration of the peptides into C2C12 myoblast cells was successful (Fig. 1.)and the peptide was also incorporated into the cytosolic and nuclear fractions of the cells in a concentration-dependent manner, as determined by immunofluorescence and Western blot analyses. Interactions between SMTNL1-mimicking peptides and PR were investigated using in vitro binding assays and demonstrated specific binding of the TAT-SMTNL1-mimicking peptide selectively to PR-B (Fig. 2.). In a model of insulin resistance in skeletal muscle cells with high glucose and insulin concentrations, we observed a decrease in insulin receptor-ß (IR-ß) and IRS-1 expression and Akt/PKB enzyme activity, and an increase in IRS-1Ser612/Ser1101 phosphorylation (Fig. 3.). The insulin sensitizing effect of the TAT-SMTNL1-mimicking peptide was demonstrated by significantly counteracting the above-mentioned changes in C2C12 myoblast cells. The peptide affected the glucose transport and also increased the extracellular acidity rate assayed by Seahorse cell metabolic analyser. The docking site of SMTNL1-mimickig peptide to PR-B is also investigated by molecular dynamic analysis in collaboration with the Hetényi lab.

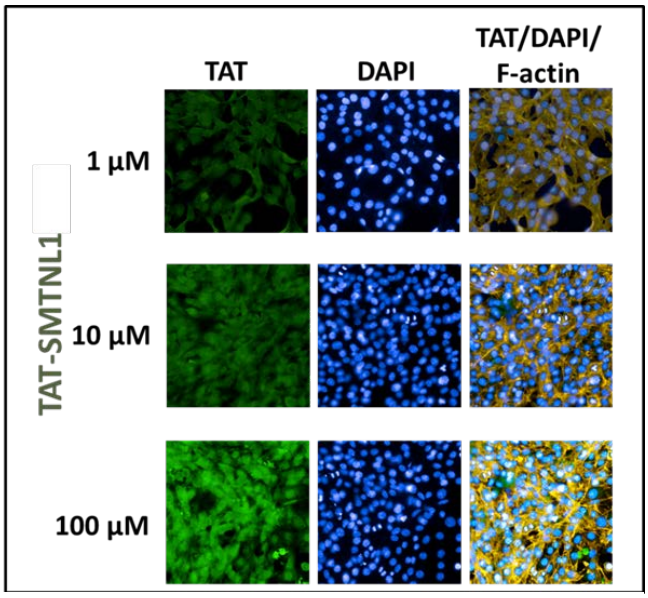

Figure 1. Concentration-dependent penetration of the SMTNL1-mimicking peptide (green) co-stained with DAPI nuclear stain.

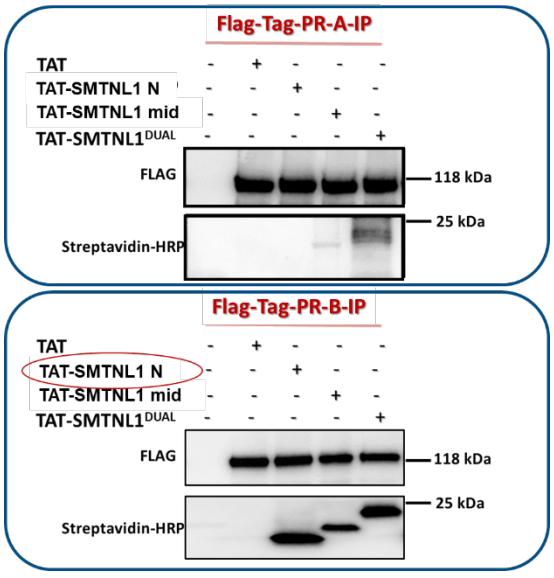

Figure 2. The SMTNL-1 mimicking peptide selectively binds the progesterone receptor B isoform.

Although the experiments are completed but due to a possible patenting, publications are on hold till the end of this year.

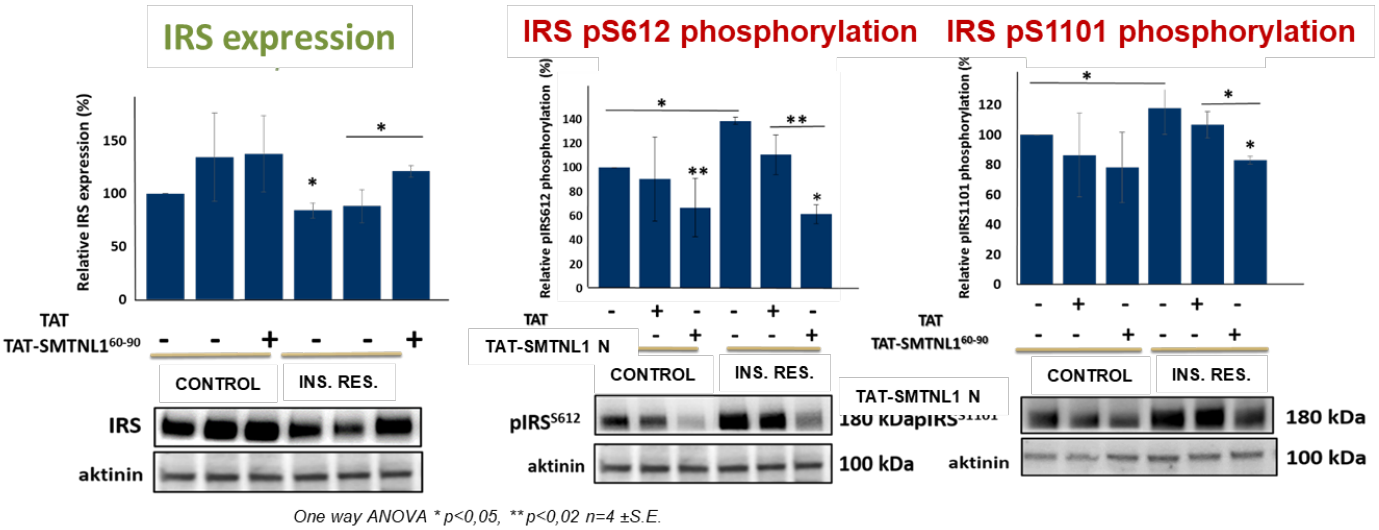

Figure 3. The TAT-SMTNL1-mimcking peptide promotes insulin sensitivity by decreasing the phosphorylation of Ser residues (Ser612 and 1101) of IRS1 in insulin resistant model in differentiated C2C12 cells.

Myosin phosphatase: Unexpected functions of a long-known enzyme. *Kiss A, Erdődi F, Lontay B.* Biochim Biophys Acta Mol Cell Res. 2019 Jan;1866(1):2-15. doi: 10.1016/j.bbamcr.2018.07.023. Epub 2018 Aug 2.

Work package 2: Investigation of the role of SMTNL1 in the insulin signaling pathway

SMTNL acting as a cofactor on PR-B regulates the gene expression of numerous enzymes playing a role in carbohydrate and lipid metabolism and that of those enzymes modulating the activity of them. Insulin resistance (InR) is manifested in skeletal muscle by decreased insulin-stimulated glucose uptake due to impaired insulin signaling and multiple post-receptor intracellular defects. Chronic glucose-induced insulin resistance leads to the activation of Ser/Thr kinases and elevated phosphorylation of insulin receptor substrate 1 (IRS1) on Ser residues. We provided evidence for the insulin-sensitizing role of smoothelin-like protein 1 (SMTNL1) that is a ligand-dependent co-regulator of steroid receptors, predominantly the progesterone receptor. SMTNL1 was transiently overexpressed in insulin-resistant C2C12 myotubes. A proteome profiler array revealed that mTOR and Ser/Thr kinases were SMTNL1-dependent signaling pathways. In the presence of progesterone, overexpression was coupled to decreased Ser phosphorylation of IRS1 at Ser307, Ser318, and Ser612 residues. SMTNL1 also induced the expression and activity of the

p85 subunit of PI3K. SMTNL1 regulated the expression of PKCε, which phosphorylates IRS1 at Ser318 residue. SMTNL1 also regulated ERK1/2 and JNK, which phosphorylate IRS1 at Ser612 and Ser307, respectively. Real-time metabolic measurements of oxygen consumption rate and extracellular acidification rate revealed that SMTNL1 improved glycolysis and promoted the utilization of alternative carbon fuels. SMTNL1 also rescued the mitochondrial respiration defect induced by chronic insulin exposure. Collectively, SMTNL1 plays a crucial role in maintaining the physiological ratio of Tyr/Ser IRS1 phosphorylation and attenuates the insulin-signaling cascade that contributes to impaired glucose disposal, which makes it a potential therapeutic target

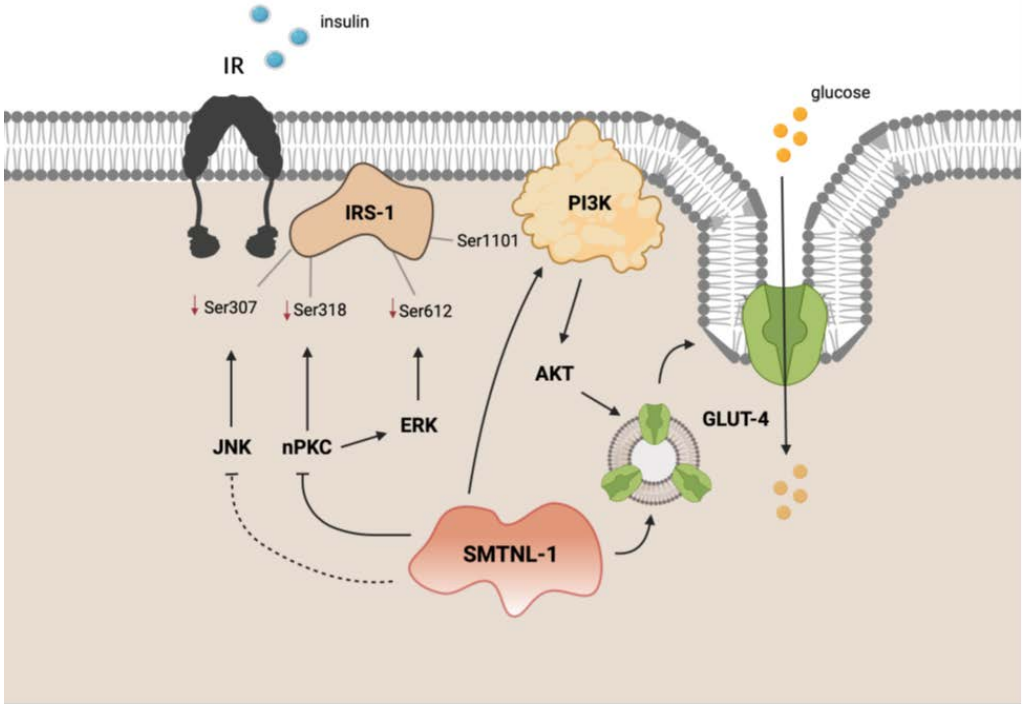

Figure 4. Insulin sensitizing effects of SMTNL1 in InR C2C12 myotubes. *SMTNL1 decreased PKCε gene expression, which hampered the activity of ERK1/2 leading to a decrease in IRS1 Ser612 phosphorylation. Ser612 phosphorylation initiates the PI3K association, activates the PI3K-Akt-mTOR pathway, and downregulates JNK activation, leading to a decreased IRS1 Ser307 phosphorylation. SMTNL1 promotes glucose uptake via activation of Akt and AMPK pathways and by inducing GLUT4 expression.*

for improving InR (Fig.4.).

We have also investigated the role of SMTNL1 in another insulin resistant model, the hyperthyroid human samples and C2C12 cells cultures. Hyperthyroidism triggers a glycolytic shift in skeletal muscle (SKM) by altering the expression of metabolic proteins, which is often accompanied by peripheral insulin resistance. Our aim was to elucidate the role of SMTNL1 in SKM under physiological and pathological 3,3′,5-Triiodo-L-thyronine (T3) concentrations.

Human hyper- and euthyroid SKM biopsies were used for microarray analysis and proteome profiler arrays. Physiological and supraphysiological concentrations of T3 were applied on differentiated C2C12 cells upon SMTNL1 overexpression to assess the activity and expression level of the elements of insulin signaling and glucose metabolism. Mitochondrial respiration and glycolysis were measured to determine cellular metabolic function/phenotype of our model system in real-time. Expression of genes related to energy production, nucleic acid- and lipid metabolism were changed significantly in hyperthyroid samples. The phosphorylation levels and activity of AMPKα2, ERK1/2 and JNK were increased in the hyperthyroid samples compared to control. SMTNL1 expression was decreased in the hyperthyroid samples and in T3-treated C2C12 cells. Moreover, it selectively regulated TRα expression at the transcriptional level. T3 excess triggered the development of insulin resistance while overexpression of SMTNL1 induced insulin sensitivity through the inhibition of JNK activity and hampered the non-genomic effects of T3 by decreasing the activity of ERK1/2 through PKCδ. SMTNL1 overexpression reduced IRS Ser phosphorylation in hyperthyroid model to restore the normal responsiveness of glucose transport to insulin (Fig.5.).

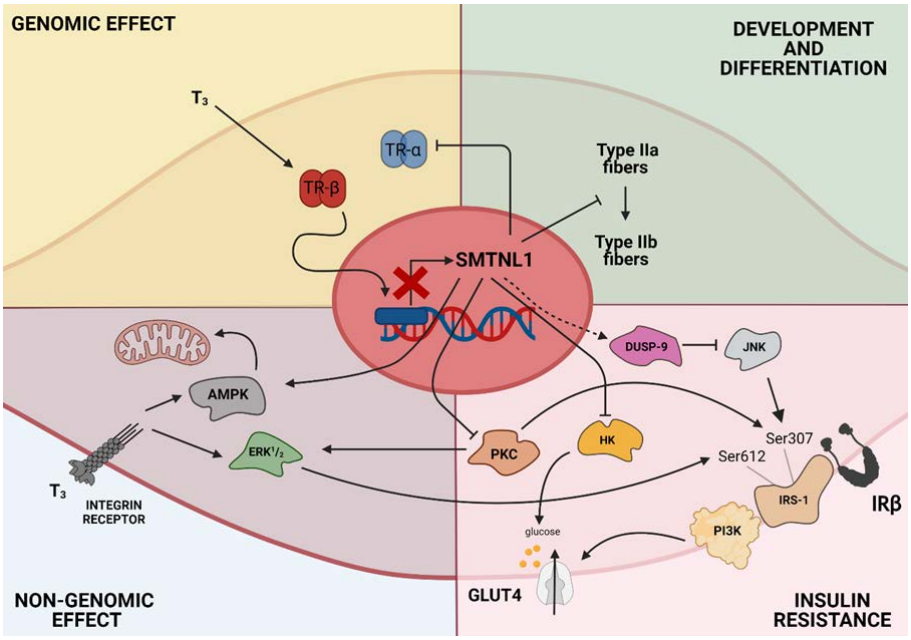

Figure 5. SMTNL1 Maintaining the Homeostasis of SKM in Hyperthyroidism SMTNL1 potentially prevents hyperthyroidism-induced changes in SKM via the following mechanisms: *1) SMTNL1 compensates for the glycolytic phenotype shift of SKM triggered by pathological T3 exposure; 2) SMTNL1 induces insulin sensitivity through an insulin-independent heterologous pathway by inhibiting the activity of JNK; 3) SMTNL1 hampers the non-genomic effect of T3 by decreasing the activity of ERK1/2 through PKCδ; 4) SMTNL1 regulates glucose phosphorylation and balances glycolysis and glycogen synthesis by the regulation of HK II; and 5) SMTNL1 selectively inhibits TRα expression, which is a key target of insulin-dependent signaling governed by T3.*

SMTNL1 regulated glucose phosphorylation and balances glycolysis and glycogen synthesis via the downregulation of hexokinase II. T3 overload strongly increased the rate of lactate production, while SMTNL1 overexpression antagonizes the T3 effects. These lines of evidence suggest that SMTNL1 potentially prevents hyperthyroidism-induced changes in SKM and it holds great promise as a novel therapeutic target.

The pathological elevation of the active thyroid hormone (T_3) level results in the manifestation of hyperthyroidism, which is associated with alterations in the differentiation and contractile function of SKM. Myosin phosphatase (MP) is a major cellular regulator that hydrolyzes the phosphoserine of phosphorylated myosin II light chain. MP consists of an MYPT1/2 regulatory and a protein phosphatase 1 catalytic subunit. Smoothelin-like protein 1 (SMTNL1) is known to inhibit MP by directly binding to MP as well as by suppressing the expression of MYPT1 at the transcriptional level. Supraphysiological vs. physiological concentration of T_3 were applied on C2C12 myoblasts and differentiated myotubes in combination

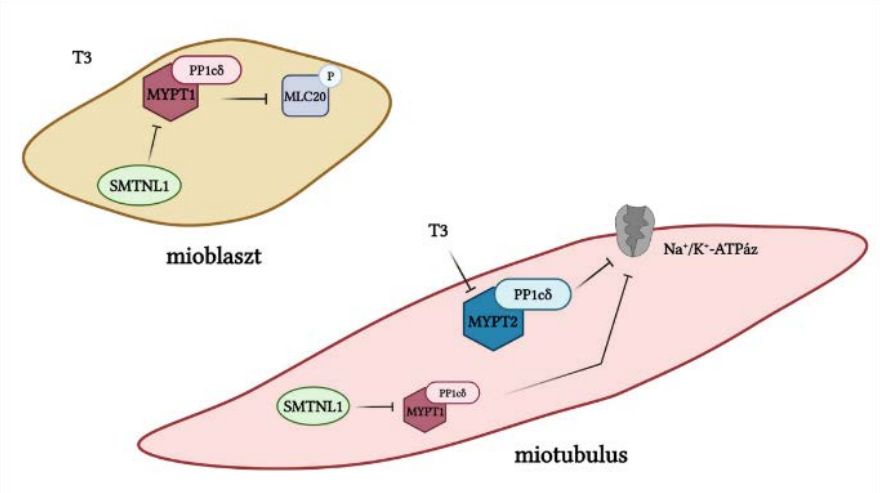

Figure 6. *In non-differentiated myoblasts, MP is in complex with MYPT1 and its expression and activity is regulated by SMTNL1 that modulates the MLC20 phosphorylation, cytoskeletal remodeling and migration. In myotubes, where the action of MP is less profound in the regulation of muscle contractility, the MP holoenzyme PP1c is predominantly complexed with MYPT2. MYPT2 expression is stimulated by T_3, and MP is regulated by the T_3-induced ROCK phosphorylation. MP acts as a possible regulator of NKA by dephosphorylating its inhibitory phosphorylation sites.*

with the overexpression of SMTNL1 to assess the role and regulation of MP under these conditions. In non-differentiated myoblasts, MP included MYPT1 in the holoenzyme complex and its expression and activity was regulated by SMTNL1, affecting the phosphorylation level of MLC20 assessed using semi-quantitative Western blot analysis. SMTNL1 negatively influenced

the migration and cytoskeletal remodeling of myoblasts measured by high content screening. In contrast, in myotubes, the expression of MYPT2 but not MYPT1 increased in a T_3-dependent and SMTNL1-independent manner. T_3 treatment combined with SMTNL1 overexpression impeded the activity of MP. In addition, MP interacted with Na^+/K^+-ATPase and dephosphorylated its inhibitory phosphorylation sites, identifying this protein as a novel MP substrate. These findings may help us gain a better understanding of myopathy, muscle weakness and the disorder of muscle regeneration in hyperthyroid patients (Fig.6.).

Major E, Győry F, Horváth D, Keller I, Tamás I, Uray K, Fülöp P, Lontay B. Front Endocrinol (Lausanne). 2021 Oct 5;12:751488. doi: 10.3389/fendo.2021.751488. eCollection 2021. PMID: 34675885 Free PMC article.

Smoothelin-Like Protein 1 Regulates the Thyroid Hormone-Induced Homeostasis and Remodeling of C2C12 Cells via the Modulation of Myosin Phosphatase. *Major E, Keller I, Horváth D, Tamás I, Erdődi F, Lontay B.* Int J Mol Sci. 2021 Sep 24;22(19):10293. doi: 10.3390/ijms221910293.

Mechanisms by which smoothelin-like protein 1 reverses insulin resistance in myotubules and mice. *Tamas I, Major E, Horvath D, Keller I, Ungvari A, Haystead TA, MacDonald JA, Lontay B.* Mol Cell Endocrinol. 2022 Jul 1;551:111663. doi: 10.1016/j.mce.2022.111663. Epub 2022 May 1.

Work package 3: The role of SMTNL1 in reproductive disorders of women with insulin resistance: implantation and endometrial development

The incidence of infertility is significantly higher in women displaying diseases linked to impaired glucose homeostasis, such as insulin resistance. The defect of glucose metabolism is proved to interfere with the process of healthy fertilization, however, the molecular mechanism of the underlying causes are yet to be uncovered. *Smtnl1* null mice exhibits a reproductive phenotype and we hypothesized that infertility, high embryonal lethality and reproductive disorders are related to SMTNL1. We have found that SMTNL1 regulates the gene expression of insulin-sensitive elements of endometrium. It also promotes the differentiation of primary endometrial stromal cells (ESC) to decidualized cells. In addition, SMTNL1 modulated the myosin phosphatase activity resulting in altered migration and cytoskeletal properties of the smooth muscle and epithelial cells responsible for the implantation of trophoblasts.

In vitro human endometrial model was established to investigate the effect of SMTNL1 in pregnant (medoxyprogesterone treatment) and in gestational diabetes (GDB) applying hyperglycaemic-hyperinsulinaemic conditions. In endometrial epithelial (Ishikawa) and decidualised human endometrial stromal cells (HESC) SMTNL1 overexpression evoked controversial effect on the migration capacity of Ishikawa cells, on a progesterone-dependent manner (Fig. 7.). SMTNL1 had

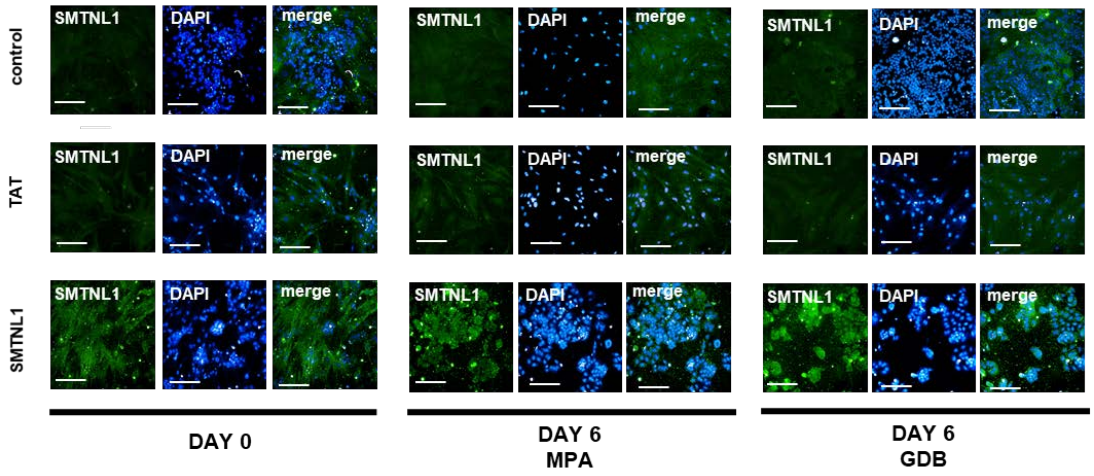

Figure 7. SMTNL1 (green) promoted the differentiation of human endometrial stomal cells assayed with DAPI (blue) nuclear co-stain. *Pregnancy (MPA) increased the differentiation and gestational diabetes (GDB) hampered the decidualization.*

no significant effect on the gene expression of the elements of insulin signaling pathway, such as IRS-1, Akt and ERK1/2 but their phosphorylation levels and activity were altered through the regulation of the gene expression of the novel type protein kinase C ε (nPKCε). Moreover, SMTNL1 mimicking peptide was introduced to HESC culture. Secreted prolactin levels were

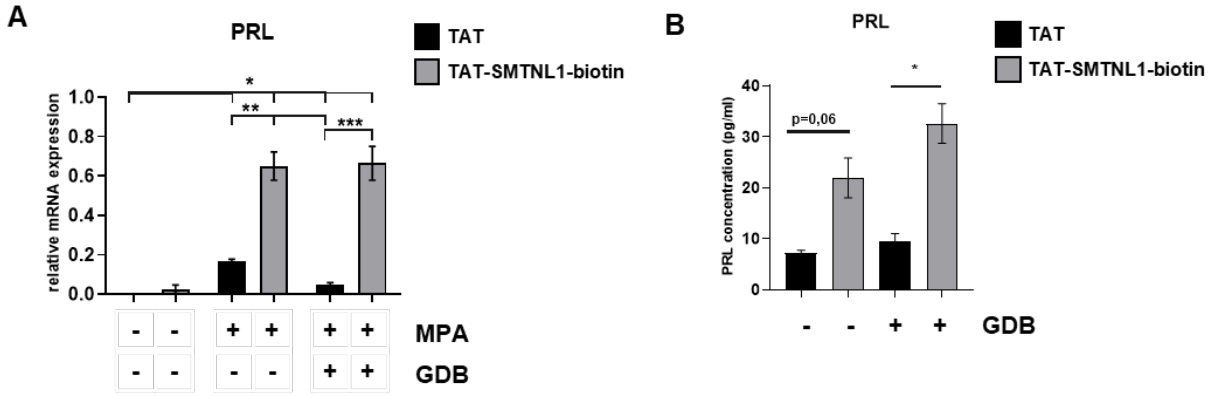

Figure 8. SMTNL1 positively regulate the expression of endometrial prolactin (PRL). *The SMTNL1-miicking peptide increased the PRL mRNA (A) and protein (B) expression and rescue the effect of gestational diabetes (GDB) compared to TAT control peptide treatment.*

examined by ELISA technique, and significant elevation of prolactin upon the peptide application was registered. We validated our results by RT-PCR, checking several differentiation (PRL and IGFBP-1) (Fig. 8.) and cytokine (IL-6) mRNA levels. It collectively suggests the insulin-sensitizing effect of SMTNL1 in epithelial cells and a potential target to improve fertility disorders through the regulation of decidualization and prolactin secretion.

SMTNL1 overexpression and the application of SMTNL1-miicking peptides also influence the insulin signaling pathway and migratory capacity of the epithelial cell layer of the endometrium

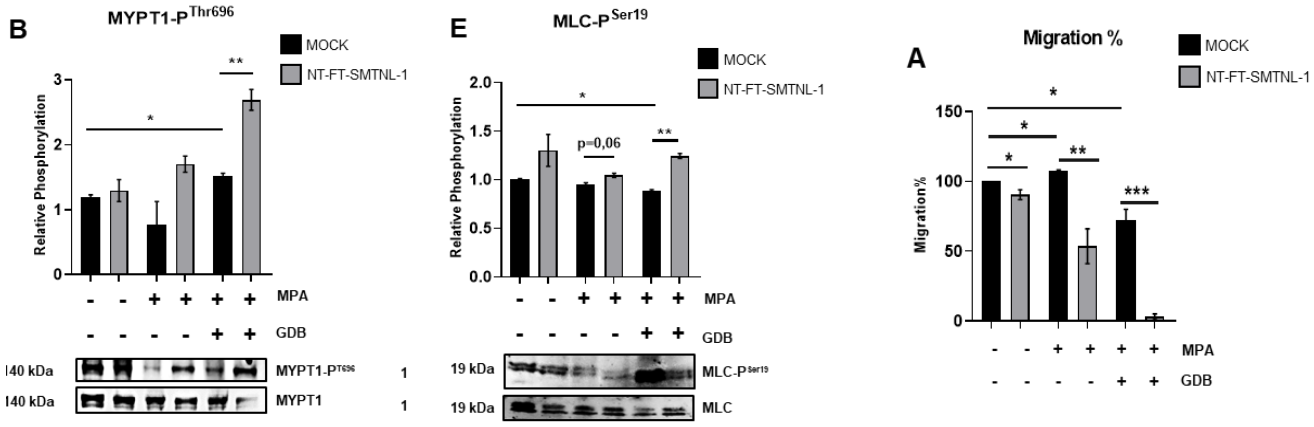

Figure 9. SMTNL1 increased the inhibitory phosphorylation of the MYPT1 regulatory subuit of the myosin phosphatase holoenzyme that regulates the myosin light chain phosphorylation resulting in a decrease in migration capacity. *In vitro physiological pregnancy (MPA) and gestational diabetes (GDB) model and SMTNL1 overexpression influence the expression and phosphorylation of MYPT1 (B) and MLC20 in Ishikawa cells. Migration capacity was also assayed during a 24 hours scratch assay (A). Ishikawa cells were transfected with empty vector or NT-FT-SMTNL1. Protein samples from whole-cell lysates were analyzed using Western blot with anti-MYPT1-P^{T696} (**B**), anti-MLC-P^{Ser19} (**E**) Values represent n = 3-6, mean ± SEM. Groups were compared using unpaired two-tailed t-tests, p<0.05 compared to control/mock (*), p<0.05 compared to MPA/mock (**), p<0.05 compared to GDB/mock (***)Scratch assay of SMTNL1 overexpressed Ishikawa cells in in vitro physiological pregnancy (MPA) and gestational diabetes (GDB) model. Ishikawa cells were stained and transfected with empty vector or NT-FT-SMTNL1 (A).*

modelled by Ischikawa cells. SMTNL1 increased the phophorylation of the MYPT regulatory subunit of myosin phosphatase leading to the decrease of its activity. It indirectly affacted the phosphorylation state of the myosin light chain resulting in a decreased phosphorylation of its regulatory Ser phosphorylation site. These results are correlated with the migration ability of Ischikawa cells assay by scretch assay. Based on our results, the physiological function of the inner endometrial layer of the endometrium, the entry gate of trophoblast cells are also regulated by

SMTNL1, and the application of the SMTNL1-mimicking peptide can help to maintain normal cytoskeletal function and implantation.

The significance of our research is a potent PR-B selective regulatory peptide which can act on

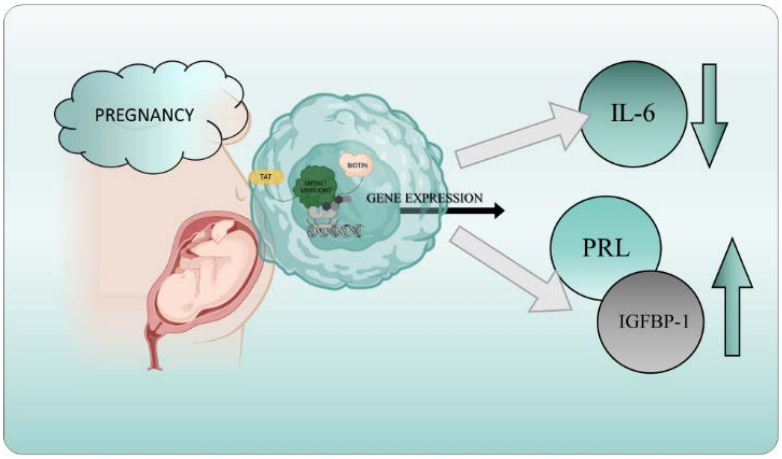

Figure 10. SMTNL1 promotes reproductive functions by downregulating interleukin 6 and increasing prolactin and differentiation marker IGFBP-1 protein expression.

gene expression governed by progesterone. Mimicking the coregulatory action of SMTNL1, it can result in a global gene expression regulation and can shift the insulin resistant state to an insulin sensitive one. Since PR-B mediates reproductive responses to progesterone in a tissue-selective manner and PR-A and PR-B isoforms are functionally distinc mediators of progesterone action *in vivo.* The selective inhibitor of PR-B does not influence the physiological effect of progesterone through PR-A. The SMTNL1-mimicking peptide improves on isulin resistance in skeletal muscle and endmometrial cells regulating the global metabolism and the reproductive function, respectively (Fig. 10.).

Ilka Keller M.D[1], Ádám Ungvári[1], Evelin Major[1], István Tamás[1], Dániel Horváth[1], Beáta Lontay[1] *SMTNL-1 regulates the insulin signalling pathway in in vitro gestational diabetes model of human endometrial epithelium,* Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary [1] (under review)

Ilka Keller M.D[1], Ádám Ungvári[1], Evelin Major[1], Reem Khamis, Fanni Szalmas, Beáta Lontay[1] *Membrane-permeable SMTNL-1mimicking peptide promotes insulin sensitivity in insulin*

resistant skeletal muscle cells, Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary [1](under review)

Publications and presentation:

Our research team published four papers (all Q1 papers) connected to the topic of the work plan and two additional papers are submitted and waiting for decision (Ilka Keller M.D [1], Ádám Ungvári [1], Evelin Major [1], István Tamás [1], Dániel Horváth [1], Beáta Lontay [1] SMTNL-1 regulates the insulin signalling pathway in in vitro gestational diabetes model of human endometrial epithelium, *Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary [1])* and (Ilka Keller M.D [1], Ádám Ungvári [1], Evelin Major [1], Reem Khamis, Fanni Szalmas, Beáta Lontay [1] Membrane-permeable SMTNL-1miicking peptide promotes insulin sensitivity in insulin resistant skeletal muscle cells, *Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary [1]).* One more paper is under preparation. Another six paper not related to the grant has been published but cited NKFIH. Two PhD students graduated related to the grant (2019 and 2022) and five invited talk were given at international conferences. 13 TDK students given talks and 6 of them promoted to the OTDK.