TNF-related weak inducer of apoptosis as a regulator of β-oxidation and differentiation in murine adipocytes

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Introduction

Obesity is known to be strongly correlated with immunodeficiency. which is particularly problematic for obese cancer patients with cancers typically responsive to immunotherapy. Ways to circumvent obesity-linked immunodeficiency are vital in cancer treatment for such individuals.

A promising approach to combating obesity-induced immunodeficiency is through reducing obesity itself through medication. Another approach is through reintroducing cytokines involved in activation, differentiation, or growth of immune cells.

Tumor necrosis factor (TNF) related weak inducer of apoptosis, also known as TWEAK or TNFSF12, is such a cytokine. It not only has downstream signaling pathways that promote immune cell proliferation/determination but also has large-sweeping effects as an inflammatory cytokine of the TNF family; thus, it is a therapeutic agent of interest in the low-grade inflammation present in obesity.

Moreover, obesity research often involves rat adipocyte models, for which there are straightforward differentiation cocktails to utilize. These consist of 3 main molecules, which bind to receptors that induce transcription of adipogenic genes. Many papers have started to use another differentiation agent, Rosiglitazone, to speed up the process; however, studies comparing differentiation levels with and without are inconclusive or suggest no difference.

Thus, we aim to observe the effects of TWEAK in a murine adipocyte models to determine if it has any lipolytic and anti-differentiation effects that would cause anti-obesity effects.

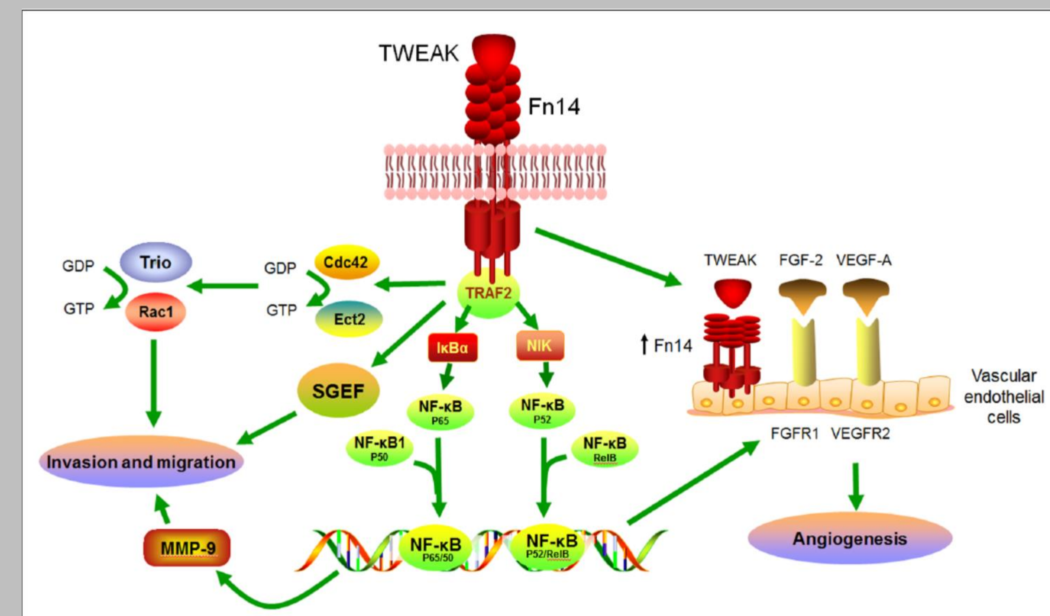


Figure 1. (Hu, et al., 2017, pg. 3)

Hypotheses

1. TWEAK antagonizes differentiation and growth of pre-adipocytes and adipocytes
2. TWEAK will be expressed at lower levels in obese mice as compared to those at normal weights
3. Rosiglitazone and other thiazolidinediones are not effective in promoting differentiation

Methodology

Cell culture: 3T3-L1 pre-adipocyte cells were sourced from ATCC (ID: CL-173). Cells culture medium comprised of 10% Bovine Calf Serum and 90% DMEM with 4.5 g/L glucose as per ATCC recommendation. The cells were cultured in 100 mm culture plates in a cell culture incubator set to 37 degrees Celsius and 5% CO_2. Cells were frozen immediately upon arrival for 1 week, then thawed and subcultured near confluency.

Oil Red O Staining: Oil Red O staining was done using a StatLab kit (ID: KTORO) . Stained cells were cultured in *Lab-Tek II Chamber Slides.* After medium was aspirated, plates were washed with PBS, fixed with 10% formalin, then stained with Oil Red O solution and modified Mayer's Hematoxylin solution. Slides are then removed from chamber and observed; photos of slides were taken on an Olympus microscope with digital camera.

RT-qPCR (primer selection): Target genes were decided through literature analysis intended to find markers of adipocyte differentiation, fatty acid synthesis, β-oxidation, and lipolysis that are widely expressed 3T3-L1 cells or white adipocyte tissues. Primers were found on PrimerBank, and NCBI PrimerBlast was used to determine specificity. β-actin was the housekeeping/reference gene used.

Differentiation: Two differentiation protocols were utilized to assess efficacy of thiazolidinediones in adipocyte differentiation. Thiazolidinediones like are not present in ATCC protocols but are becoming more popular in adipocyte differentiation. Differentiation medium 1 is 90% DMEM, 10% FBS, 1.0 μM Dexamethasone, 0.5 mM IBMX, and 1.0 μg/mL bovine insulin. Differentiation medium 2 follows Sigma-Aldrich protocols: 90% DMEM/F12 (1:1), 10% FBS, 1.0 μM Dexamethasone, 0.5 μM IBMX, 1.0 μg/mL bovine insulin, and an added 1 μM Rosiglitazone, a thiazolidinedione PPARγ activator. After a differentiation period of 48 hours adipocyte growth medium is used. Growth medium 1 is 10% FBS, 90% DMEM, and 1 μg/mL insulin, and growth medium 2 is 1 is 10% FBS, 90% DMEM/F12, and 1 μg/mL insulin. TWEAK was added to the cells on the 10th day of differentiation, and analysis was done on the 13th day. 4 6-well plates and 4 *Lab-Tek II Chamber Slides* were differentiated using Protocol 2. 2 6-well plates, along with 4 Lab-Tek II Chamber Slides, were differentiated using Protocol 1.

TWEAK ELISA assay: Done using Invitrogen Mouse TWEAK ELISA kit (ID: EMTNFSF12). Samples were collected from mice aged 2 months. Blood serum samples were obtained from 3 control mice on a chow-diet and 3 obese mice on NASH diet. 50 μm serum was collected per mouse, and serum was diluted 10-fold with diluent provided by the kit. 100 μL of dilute sample and dilute TWEAK standard protein was added to appropriate wells and left to incubate overnight; following was the addition of a biotin conjugate, then Streptavidin-HRP, and TMB substrate with incubation and washing between each step. At the end, stop solution was added without washing of the plate, and the plate's absorbance was read at 450 nm.

Results

Figure 1: Pretreated/Prestained 3T3-L1 fibroblasts:

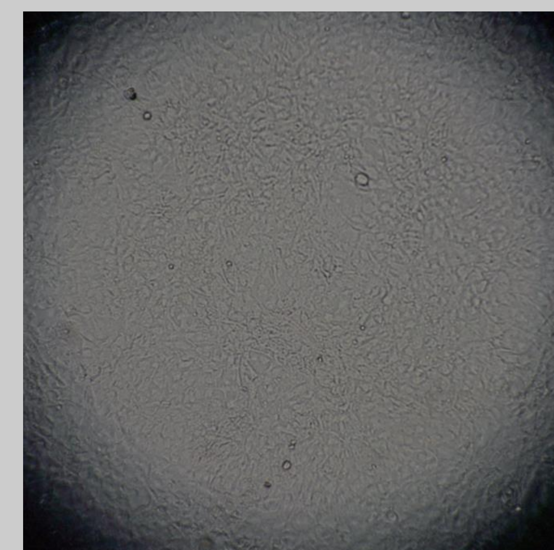


Figure 2: Protocol 1 Adipocytes (-Rosiglitazone):

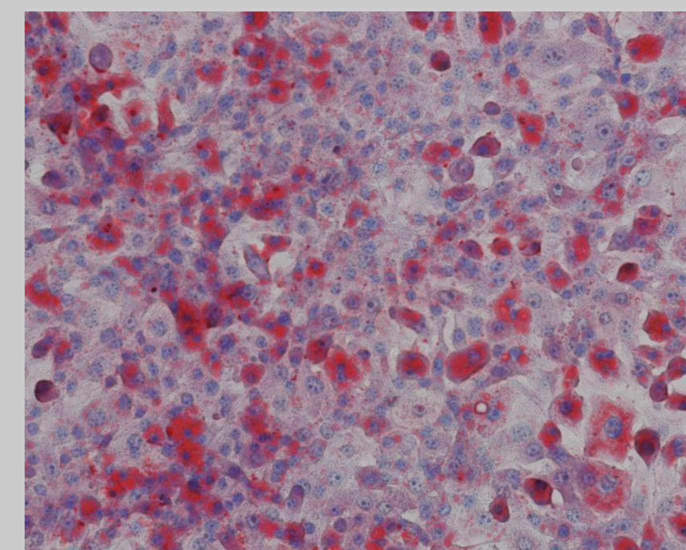


Figure 2: Protocol 2 Adipocytes without TWEAK treatment:

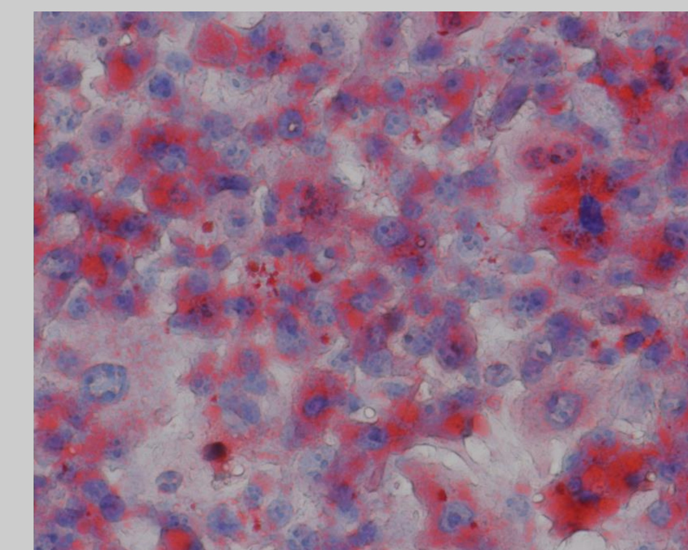


Figure 4: Cocktail 2 Adipocytes with TWEAK treatment

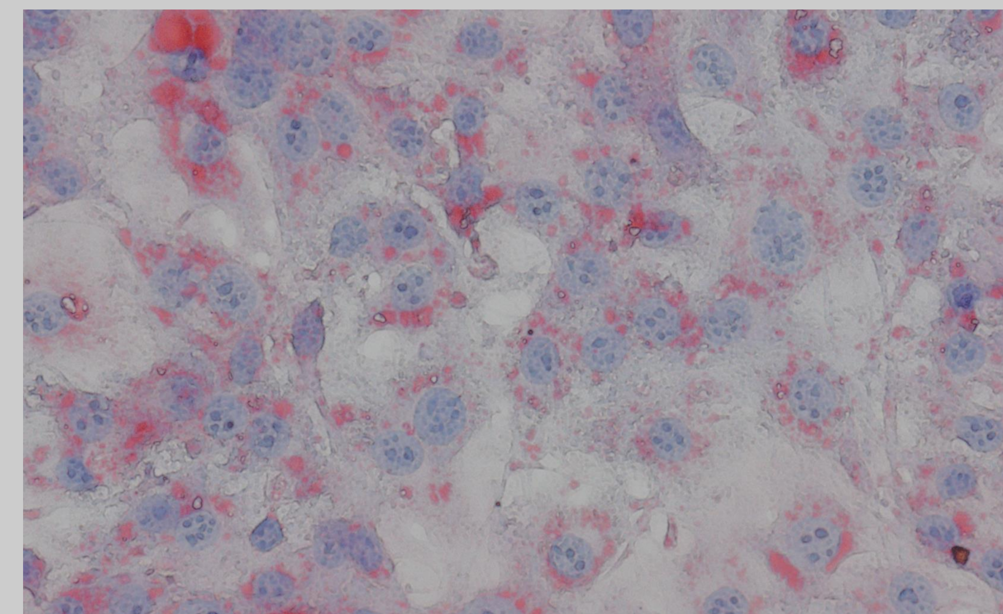
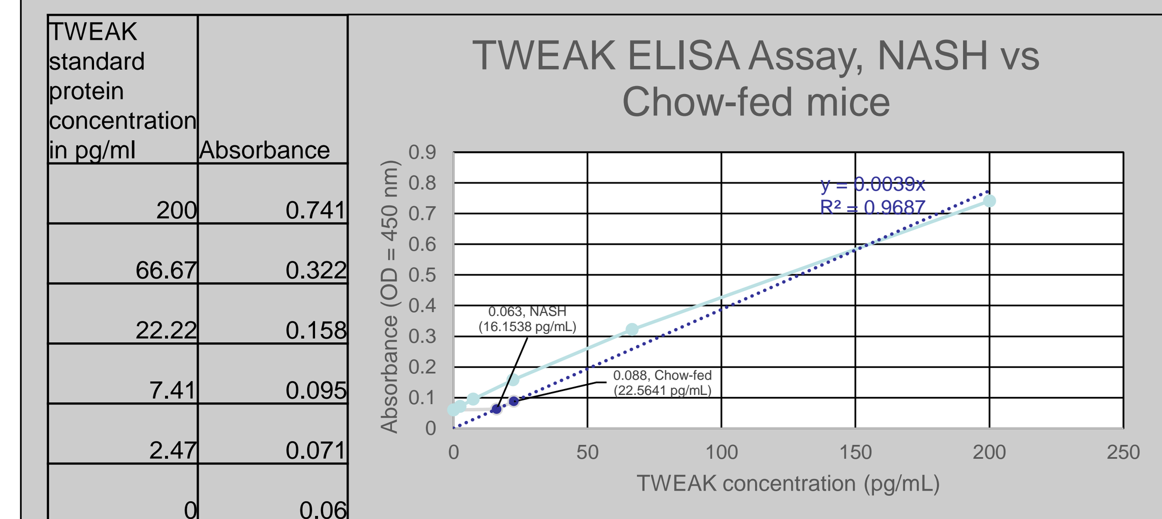


Figure 5: RT-qPCR primers designed for TWEAK treated cells:

Gene Target Name	Forward Primer	Reverse Primer
β-Actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
PPARγ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
PPARα	AACATCGAGTGTCGAATATGTGG	CCGAATAGTTCGCCGAAAGAA
ACACA	CTCCCGATTCATAATTGGGTCTG	TCGACCTTGTTTTACTAGGTGC
Leptin	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
ADIPOR 1	AATGGGGCTCCTTCTGGTAAC	GGATGACTCTCCAACGTCCCT
ADIPOR 2	GCCAAACACCGATTGGGGT	GGCTCCAAATCTCCTTGGTAGTT
SREBP-1C	TGACCCGGCTATTCCGTGA	CTGGGCTGAGCAATACAGTTC
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
HSL	GATTTACGCACGATGACACAGT	ACCTGCAAAGACATTAGACAGC

Results (cont.)



TWEAK standard protein concentration in pg/ml	Absorbance
200	0.741
66.67	0.322
22.22	0.158
7.41	0.095
2.47	0.071
0	0.06

ELISA standard protein results were graphed along with linear regression of the standard curve. The absorbances of the NASH and Chow-fed mice were then plotted on the regression line; since they were diluted 10-fold, the real concentrations are 161.538 pg/mL in NASH mice and 225.641 pg/ml in chow-fed mice. The two groups significantly differ, with higher concentrations seemingly present in chow-fed mice.

Through oil red staining, triacylglycerol deposits in the cell become clearly visible and highlighted amongst other parts of the cell. We can use this to determine if cells have differentiated and to what degree. Untreated Protocol 2 is clearly seen to have the highest % differentiation and largest triacylglycerol level per cell. Untreated Protocol 1 is seen to have remarkably less and splotchy differentiation. Protocol 2 TWEAK treated cells have slightly rounder morphologies than their pre-differentiated fibroblast form, suggesting that differentiation had partially occurred, but TWEAK-treated adipocytes' fat storages were markedly small compared to untreated Protocol 2 and Protocol 1 adipocytes.

Conclusion

TWEAK is shown to inhibit differentiation and promote β-oxidation in 3T3-L1 pre-adipocyte cells; when comparing Protocol 2 cells treated with TWEAK to those not treated with TWEAK, the reduced red staining and more fusiform/spindle-like morphology of TWEAK-treated cells suggest that increased TWEAK promotes higher β-oxidation of existing fat stores as well as inhibiting further differentiation of pre-adipocytes. This supports our hypothesis that increased TWEAK levels would hamper differentiation.

Inversely, Protocol 2 adipocytes had higher percent differentiation and fatty acid synthesis than Protocol 1 adipocytes, as can be seen by the more rotund and more consistent morphology as well as larger lipid stores/red dyed areas in Protocol 2 cells when compared to those differentiated through Protocol 1. This demonstrates the effectiveness of thiazolidinedione agents like Rosiglitazone. This disproves our hypothesis and some current literature, but it can be rationalized due to Dexamethasone's similar mechanism of action.

Moreover, circulating TWEAK levels were shown to be decreased in the blood of obese mice as compared to normal mice. This supports our hypothesis and matches literature, which shows decreased immune activity (including levels of $CD8^+$ and NK cells, which are cells known to secrete soluble TWEAK). This makes sense, as immunodeficiency and subsequent loss of TWEAK would increase adipocyte fatty acid synthesis and pre-adipocyte differentiation.

Future Directions

Although the various effects of TWEAK in adipocytes have been laid out in this study, it is imperative that the mechanism and downstream cell signaling pathways are further researched. Indeed, if a more targetable downstream receptor or molecule exists that causes similar effects or an important downstream signaling pathway that cannot be disturbed for fear of adverse effect, it will greatly influence research on TWEAK. In that vein, the differentiated Protocol 2 cells (control and TWEAK-treated) will be sent for RNA-seq in efforts to try and elucidate possible downstream signaling pathways.

References

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