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Establishment of an in vitro model for the transition of differentiated preadipocytes to myofibroblasts to screen potential modulators for cosmetic applications

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1. Introduction

The transition of differentiated preadipocytes to myofibroblasts, called adipocyte-myofibroblast transition (AMT), is a process, in which adipocytes undergo profound morphological and biochemical changes resulting in a myofibroblast phenotype. Myofibroblasts are contractile, α-smooth muscle actin (α-SMA)-positive cells that produce stress fibers giving them the ability to exert contractile forces in the extracellular matrix. Together with excessive accumulation of secreted collagen fibers and other extracellular matrix proteins this leads to increased tissue stiffness. Myofibroblasts are involved in wound healing processes but are also responsible for fibrosis in different tissues including the skin [1]. They develop not only from tissue resident fibroblasts, but also from different other precursors, such as epithelial and endothelial cells, pericytes, and preadipocytes [2]. While not given much importance for a long time, myofibroblasts have received more attention in recent years in the context of potential applications for fibrosis and scarless wound healing.

The process of AMT has been described and studied only in the past decade and demonstrates a new link between adipose tissue loss and dermal fibrosis. It has been shown that cultured human subcutaneous adipocytes stimulated with profibrotic stimuli, such as transforming growth factor β (TGFβ), can change their morphology and biochemistry downregulating adipogenic markers (e.g. PPAR-γ, perilipin, FABP4, etc.), while upregulating fibrotic ones (e.g. procollagen type I, α-SMA, etc.). This results in a loss of adipocyte characteristics and a shift towards a myofibroblast phenotype [2]. While different differentiation processes for myofibroblasts originating from dermal fibroblasts have been described, the activation via the TGFβ/SMAD3 pathway leading to the production of α-SMA is the most studied one [3, 4]. There are multiple hypotheses on the triggers and exact mechanism of AMT but precise understanding of this cellular reprogramming remains unclear. Systemic metabolic stress, hypoxia and UV radiation may play a role in the induction of AMT either acting directly on preadipocytes or on mature adipocytes via different mechanisms [5, 6]. One potential mechanism includes the activation of macrophages which in turn could promote adipocyte progenitors to differentiate into myofibroblasts [5]. Indeed, factors secreted by macrophages can promote a profibrotic phenotype in human preadipocytes [7].

By the promotion of a stiff fiber-based tissue together with the loss of subcutaneous and dermal white adipose tissue, AMT could contribute to skin aging, as postulated by Wollina et al. 2017

and Kruglikov & Scherer 2016 [6, 8]. Interestingly, the process of AMT seems to be reversible providing potential as a target for therapeutic or cosmetic interventions [5]. The prevention of AMT would therefore be a potential anti-aging strategy. Thus, the aim of this study was to establish an in vitro model for AMT to be able to identify active ingredients that can protect from the consequences of this process on the skin.

2. Materials and Methods

Materials

Recombinant human TGF β 2 (n°100-35B) was purchased from PeproTech (Thermo Fisher Scientific, MA, USA). Activated macrophage-conditioned medium (AcMc) was prepared from human macrophages obtained after isolation and differentiation of blood monocytes and treated by lipopolysaccharide. RPMI and DMEM/F12 media and FBS were purchased from Dominique Dutscher (L0498-500, 702566 and 500105P1P). All components of the proadipogenic cocktail consisting of insulin, glucocorticoid, 3-isobutyl-1-methylxanthine (IBMX), and thiazolinedione were purchased from Sigma-Aldrich (MO, USA). Extracts from three different plants were received by water-based extraction of dried plant material. Plant extract C was based on monk's pepper berries (chaste tree, *Vitex agnus-castus*). For the clinical study, concentrated extract C was spray granulated on maltodextrin (Vitex Agnus Castus Extract / Vitex Agnus-Castus Fruit Extract (and) Maltodextrin (and) Aqua / Water) and formulated into a basic emulsion.

Cell culture

For the establishment of the assay, preadipocytes were isolated from human female subcutaneous adipose tissue collected after aesthetic surgery (body mass index <30 kg/m² and <45 years old) and were cultured for 24 h in 100 μ l of DMEM medium with 10% Fetal Bovine Serum (FBS) in 12-well plates then incubated in a proadipogenic cocktail. Preadipocytes treated with the basal DMEM/F12 medium were used as negative controls of the preadipocyte differentiation (undifferentiated cells). When cells were differentiated with visible lipid droplets after 7 to 10 days of culture in proadipogenic cocktail, cells were treated with human activated macrophage-conditioned medium (AcMc) for 5 days or with the recombinant human TGF β at 5 ng/ml and 2.5 ng/ml for 3 days, with or without the extracts (3 extracts at 2 concentrations), all of them were added in an adapted adipogenic cocktail. RPMI with 1% FBS was used at the same dilution as the AcMc condition as a solvent control for the AcMc treatment. The medium was changed every 2-3 days all along the period of culture. At the end of the culture, the media secretions were collected for each condition. In parallel, cells were lysed and frozen at -80°C before RNA extraction. All conditions were performed in triplicates.

RNA extraction and quantitative PCR

RNAs were extracted from the cells with the Quick-RNA MicroPrep kit (Zymo Research, CA, USA) according to the supplier's recommendations. For each condition, the three replicates of culture were pooled. The RNA concentrations were measured with a NanoDrop (ThermoFisher, MA, USA). Reverse transcription of RNA into complementary DNA (cDNA) was carried out with 500 ng of RNA, with the Reverse Transcription System kit (A3500, Promega, WI, USA), according to the supplier's recommendations. The cDNAs were diluted to obtain a final concentration of 5 ng/ μ L. Finally, the quantitative PCR was realized with 10 ng of cDNA added to a ONE Green Fast qPCR Premix (OZYA008, Ozyme, France) and 200 nM of each of the two specific primers of the targeted genes (Table 1). All the conditions were realized in duplicates. For each sample, the expression of the 18S housekeeping gene was evaluated to normalize the expression of the targeted genes. For each gene, a negative control with water

instead of cDNA was used to ensure the absence of contamination. Quantitative real-time PCR was executed with a thermocycler (7300 Real Time PCR System, Applied Biosystems, MA, USA) according to the following temperature cycles: -95°C; 10 minutes - 40 cycles: 95°C; 15 seconds then 60°C; 1 minute. The results were normalized by the CT mean of the housekeeping gene. Relative gene expression was calculated (2-deltaCT) and results were represented in fold of the respective control conditions for each time point. The mean and the standard deviation of the two values were represented.

Table 1. Primers

Gene	Sequence forward	Sequence reverse
18S	CGATGCTCTTAGCTGAGTGT	GGTCCAAGAATTTCACCTCT
PPARG2	GATACACTGCTGCAAACATATCAC	CCACGGAGCTGATCCAA
ADIPOQ	AAGGAGATCCAGGTCTTATTGG	ACCTTCAGCCCCGGGTAC
PLIN	AGTATCCCTACCTGAAGTCTGTG	CCCCTTACAGGCATAGGTATTG
FABP4	CCTTAAAAATACTGAGATTCCTCA	GGACACCCCCATCTAAGGTT
COL1A1	GGGATTCCCTGGACCTAAAG	GGAACACCTCGCTCTCCAG
ACTA2	GAAGAAGAGGACAGCACT	TCCCATTCCCACCATCAC
FN1	CAGTCCCCGGTGGCTGTCAGT	CAGCCCAACTTGGTGGCT
FGF	AGCGGCTGTACTGCAAAACGG	CCTTGATAGACACAACCTCCTCTC
PDGFRA	GACTTCGCCAAAGTGGAGGAG	AGCCACCGTGAGTTCAGAACGC

Clinical study

A randomized double-blind placebo-controlled clinical study was performed on 39 female subjects aged between 21 and 67 years (mean age: 46.3 years). The subjects were split into two groups using either a cream with 2% of the active ingredient or a corresponding placebo twice daily on the thighs. At day 0 (baseline) and after 28 and 56 days of treatment, skin elasticity and firmness were assessed using a Cutometer® dual MPA 580 probe (Courage & Khazaka, Germany). Data are shown as mean and standard deviation. Statistical analysis was performed with a Kruskal-Wallis and pairwise Wilcox test.

3. Results

Establishment of a model for AMT

To develop an in vitro model that mimics AMT, preadipocytes were isolated from human female subcutaneous adipose tissue and cultured in proadipogenic conditions. Differentiated cells were treated with either human activated macrophage-conditioned medium (AcMc) or with TGFβ at different concentrations, as both are known to induce AMT. At the end of the culture, cells were lysed, and RNA was extracted for quantitative PCR of adipogenic (PPARG2 (PPAR-γ receptor), ADIPOQ (adiponectin), PLIN (perilipin), FABP4 (adipocyte fatty acid binding protein, aP2)) and fibrogenic (COL1A1 (procollagen I), ACTA2 (alpha smooth muscle actin, α-SMA), FN1 (fibronectin) genes to assess the effect of both inducers on AMT. In addition, FGF (fibroblast growth factor) needed for myofibroblast differentiation and PDGFRA (platelet-derived growth factor receptor A), a marker of preadipocytes, were analyzed.

While the expression of adipogenic genes was very low in undifferentiated preadipocytes, the expression of these genes was increased after addition of the proadipogenic culture medium, confirming the preadipocyte differentiation (Figures 1+2). The treatment with either 2.5 ng/ml or 5 ng/ml TGFβ induced an increase in the expression of the fibrogenic genes COL1A1, ACTA2 and FN1 as well as the fibroblast growth factor (FGF) compared to the expression in differentiated preadipocytes (Figures 1+3). In contrast, the expression of all adipogenic

markers was reduced after the treatment (Figures 1+2). The preadipocyte marker PDGFRA also showed a decreased expression (Figure 1).

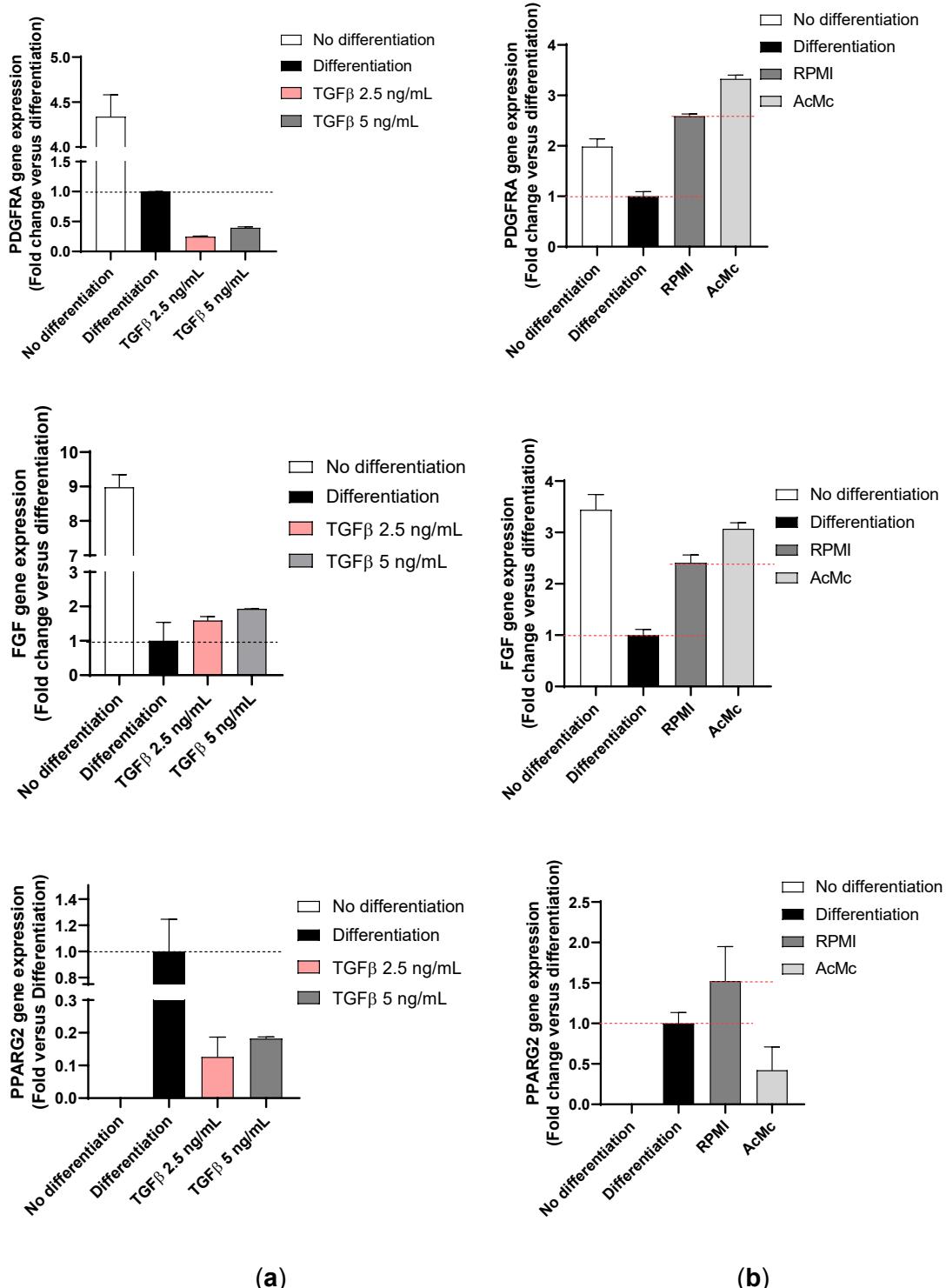


Figure 1. Gene expression of PDGFRA (platelet-derived growth factor receptor A), FGF (fibroblast growth factor) and the adipogenic marker PPARG2 (PPAR γ receptor) in differentiated preadipocytes treated with different inducers of AMT. Untreated undifferentiated and differentiated preadipocytes were used as controls. (a) Differentiated preadipocytes treated with TGF β at different concentrations; (b) Differentiated preadipocytes treated with macrophage-conditioned medium (AcMc) or the control (RPMI).

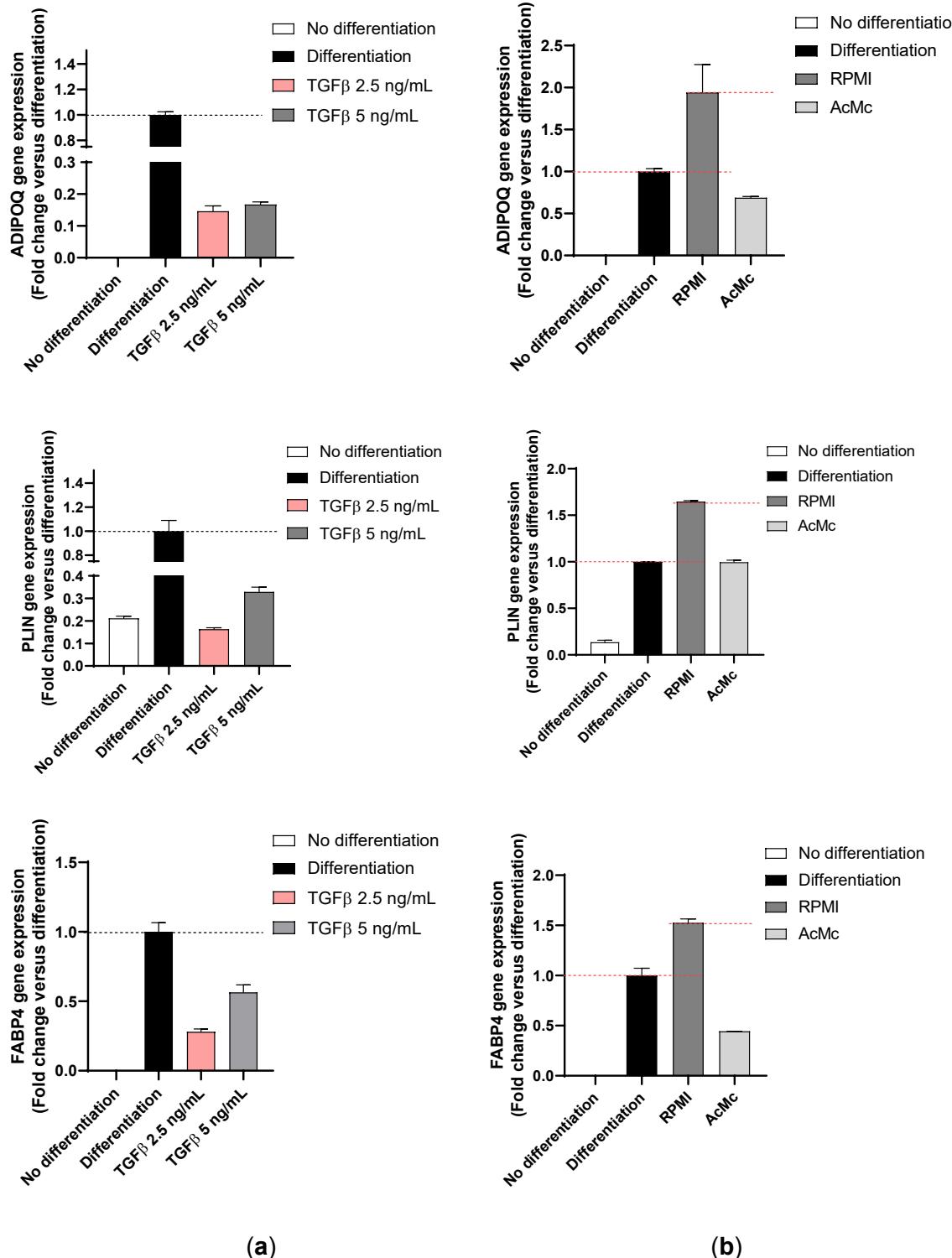


Figure 2. Expression of adipogenic genes ADIPOQ (adiponectin), PLIN (perilipin) or FABP4 (adipocyte fatty acid binding protein, aP2) in differentiated preadipocytes treated with different inducers of AMT. Untreated undifferentiated and differentiated preadipocytes were used as controls. (a) Differentiated preadipocytes treated with TGFβ at different concentrations; (b) Differentiated preadipocytes treated with macrophage-conditioned medium (AcMc) or the control (RPMI).

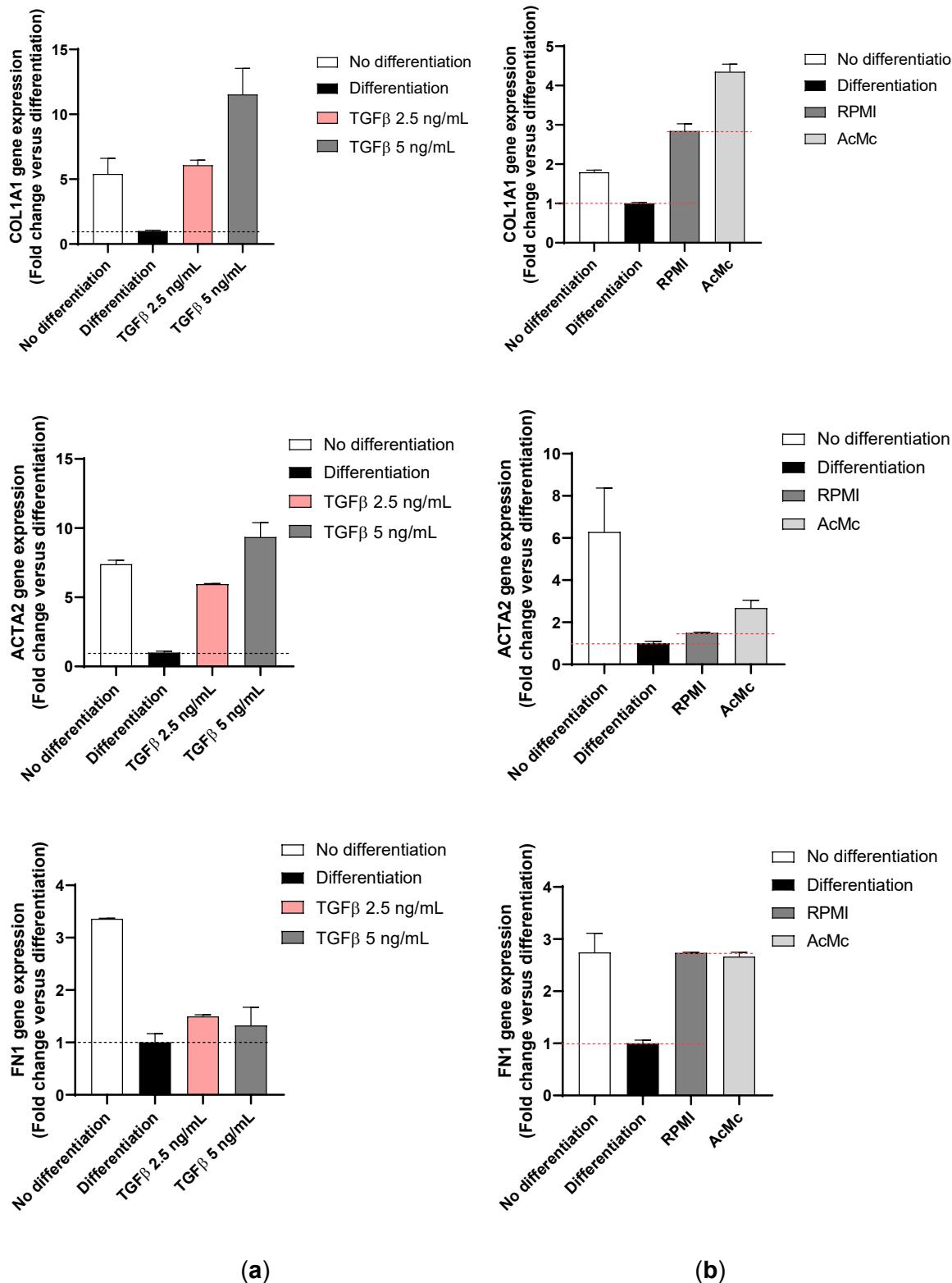


Figure 3. Expression of fibrogenic genes COL1A1 (procollagen 1), ACTA2 (alpha smooth muscle actin, α -SMA) or FN1 (fibronectin) in differentiated preadipocytes treated with different inducers of AMT. Untreated undifferentiated and differentiated preadipocytes were used as controls. (a) Differentiated preadipocytes treated with TGF β at different concentrations; (b) Differentiated preadipocytes treated with macrophage-conditioned medium (AcMc) or the control (RPMI).

In conclusion, the treatment with TGF β seemed to induce the myofibroblast phenotype on gene level. A similar effect was observed for the treatment with AcMc compared to the adipogenic condition with RPMI. The expression of all adipogenic markers was decreased (Figures 1+2), whereas the expression of fibrogenic markers was increased (Figure 3). The only exception was FN1, which did not show increased expression compared to the control condition (Figure 3B). For PLIN, the effect of AcMc was little but compared to the control RPMI, a downregulation could be observed (Figure 2B). While the potential of both TGF β and AcMc to induce AMT was confirmed, the effect of AcMc, especially on the gene expression of ACTA2 and PLIN, was less pronounced. Based on these results, 2.5 ng/ml TGF β was chosen as the most potent AMT-inducing condition and thus used in the following experiments.

Effect of three plant extracts on the modulation of AMT

Using the conditions and markers identified to have the strongest effect in the assay development step, three plant extracts (extract A-C) were tested for their modulatory effect on AMT. For this, differentiated preadipocytes were treated with 2.5 ng/ml TGF β together without (control) or with the plant extracts at different concentrations for 3 days.

The gene expression of the adipogenic marker ADIPOQ, which was decreased in cells treated with TGF β compared to untreated differentiated preadipocytes, was upregulated by the treatment with 0.1 % of extract B and 0.1 % of extract C, whereas it was downregulated by both concentrations of extract A (Figure 4A). In contrast, the expression of FABP4, which was also strongly decreased by TGF β treatment, was upregulated by treatment with 0.1 % of extract A, 0.1 % of extract B, and 0.1 % of extract C. The expression of the third adipogenic marker PPARG2, which was also downregulated by the TGF β treatment, was upregulated by treatment with 0.03 % and 0.1 % of extract B and by 0.1 % of extract C. In contrast, it was downregulated by the treatment with extract A at both concentrations (Figure 4A).

Regarding the myofibrogenic markers (Figure 4B), ACTA2, which was strongly upregulated in cells treated with TGF β , was downregulated by treatment with 0.33 % of extract A, 0.03 % of extract B or 0.1 % of extract C and upregulated by 0.1 % of extract B and 0.03 % of extract C. The expression of the fibrogenic marker COL1A1, which was strongly upregulated in cells treated with TGF β , was downregulated for all treatment conditions. In contrast, the gene expression of FN1, which was increased by treatment with TGF β , was additionally upregulated for all conditions tested (Figure 4B).

These results indicated that extract C at 0.1 % has the potential to prevent AMT. While extract B showed a similar trend, the effects of extract A were less clear and did not point to a prevention of the AMT process in preadipocytes treated with TGF β . Based on previous data on anti-aging effects obtained for extract C, we decided to continue to work with this plant extract.

Clinical efficacy of a plant extract identified to modulate AMT

Based on the in vitro data, the efficacy of the monk's pepper extract was tested in a placebo-controlled clinical study with two groups of female volunteers using either a cream containing 2% of the extract C or a corresponding placebo cream twice daily for 56 days. At the beginning and after 28 and 56 days of treatment skin elasticity and firmness were assessed. For both skin elasticity and firmness, a slight non-significant improvement was seen after 28 days of treatment with the cream containing 2% monk's pepper extract, which was not observed in the group using the placebo cream. After 56 days of treatment both parameters significantly improved in both groups with a stronger effect in the group that used the cream with monk's

pepper extract, resulting in an improvement of skin elasticity by 17% and skin firmness by 15% compared to the placebo, respectively (Figure 5).

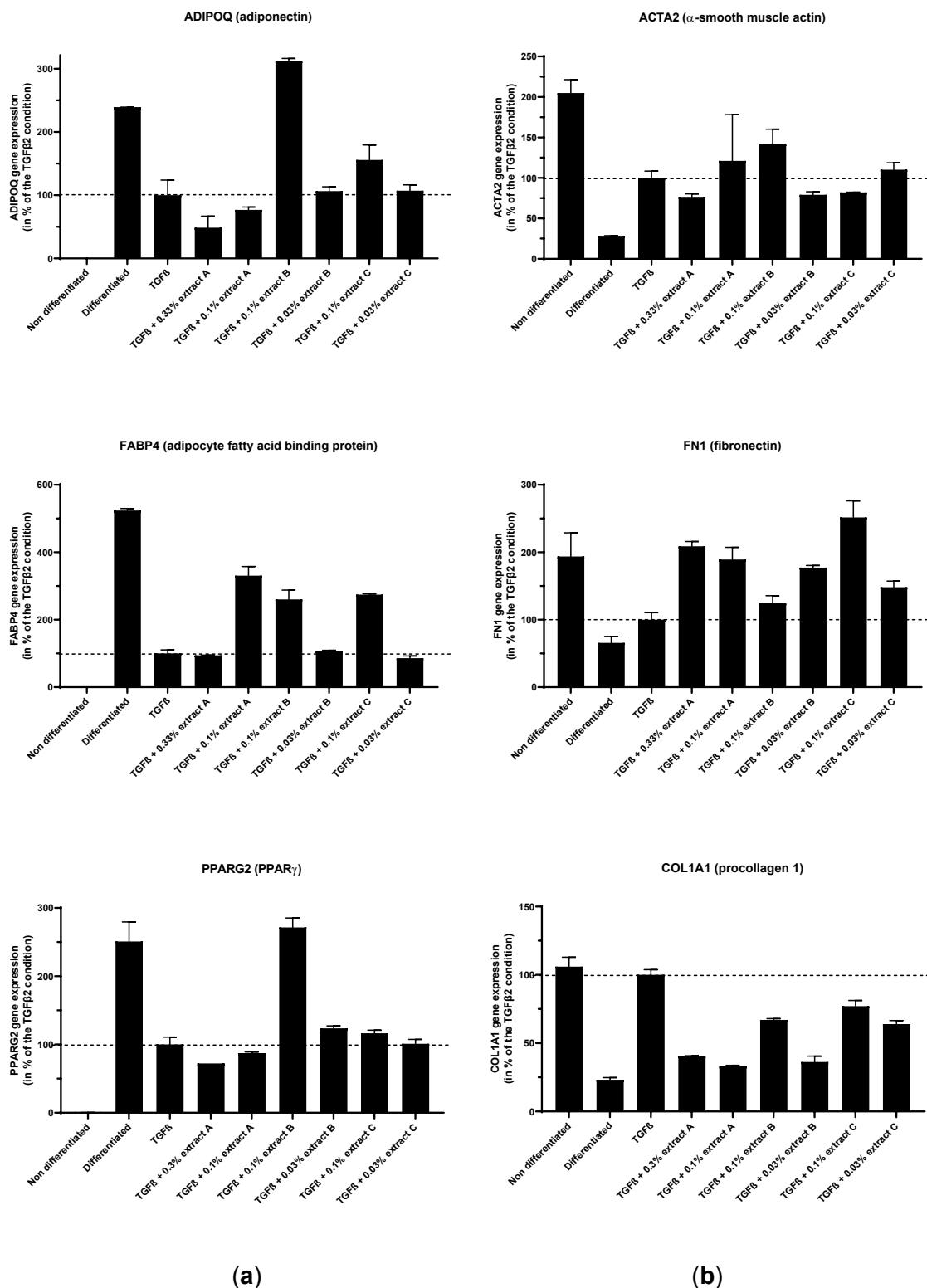


Figure 4. Gene expression of adipogenic and myofibroblast markers in differentiated preadipocytes treated with 2.5 ng/ml TGF β without (control for AMT induction) or together with three plant extracts A, B and C at different concentrations for 3 days. Untreated undifferentiated and differentiated cells served as controls. (a) Expression of adipogenic marker genes (ADIPOQ, FABP4, PPARG2) in preadipocytes. (b) Expression of myofibroblast marker genes (ACTA2, FN1, COL1A1) in preadipocytes.

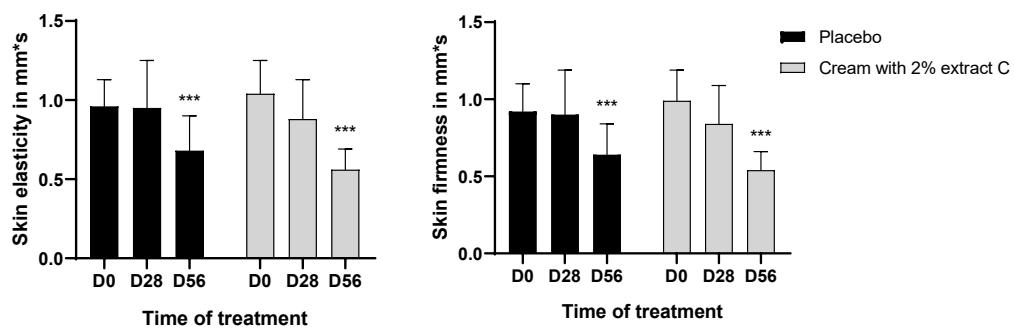


Figure 5. Skin elasticity and firmness assessed on the thighs before and after 28 or 56 days of treatment with a cream containing 2% of plant extract C or a corresponding placebo. ***p<0.001 vs. D0.

4. Discussion

In this study, a new in vitro model for the transition of dermal adipocytes to myofibroblasts was developed. We observed that both TGF β and AcMc were able to induce AMT in differentiated preadipocytes as indicated by decreased expression of adipogenic markers and increased expression of fibrogenic markers in treated cells compared to untreated cells. This is in line with previous literature [2] and confirms the potential of AcMc to induce a myofibroblast phenotype [7]. Even though the effect of AcMc was less pronounced for some of the genes, both treatments showed globally a very similar effect. PDGFRA was the only gene for which we observed a different effect by the two treatments - a slight upregulation in differentiated preadipocytes treated with AcMc and a reduction in the expression in cells treated with TGF β . PDGFRA is a gene known to be expressed more strongly in undifferentiated preadipocytes compared to differentiated adipocytes but it has also been described to be expressed together with fibrogenic genes in adipocytes which underwent transdifferentiation into myofibroblasts [9]. Many of these studies, however, were performed in mouse models.

In this context, it is also interesting to consider the involvement of dermal white adipose tissue (dWAT), a layer of white adipose tissue associated with the dermis distinct from the subcutaneous white adipose tissue (sWAT) which is located beneath the dermis. dWAT has gained increasing interest in the past 10 years and has been found to play a role in thermogenesis, immune defense, hair cycling, wound healing, fibrosis and scarring [10]. dWAT has a high degree of plasticity and can quickly undergo profound changes in the phenotype. A modulation of dWAT in aged skin has been described. Strong inflammation upon chronic UV radiation in chronically photodamaged skin displays a reduction of dWAT volume caused by a replacement of the adipose tissue with fibrotic structures [11]. Inflammatory processes may be involved as they are known to be linked to a loss of sWAT. Thus, a remodeling of dWAT by AMT could be linked with inflammaging.

The established model was used to test three plant extracts for their potential to modulate AMT. Amongst the plant extracts tested, an extract based on monk's pepper (*Vitex agnus-castus*) was able to mitigate the reduced expression of adipogenic markers (ADIPOQ, FABP4) and the increased expression of fibrogenic markers (ACTA2, COL1A1), indicating the potential to prevent the transition from dermal adipocytes to myofibroblasts. In addition, adipokines notably adiponectin (encoded by the gene ADIPOQ) has been shown to positively influence the biology of dermal fibroblasts by stimulating their production of collagen 1 and hyaluronic acid, and thus indirectly participate to matricial remodeling in the dermis [12]. While monk's pepper is well-known for its traditional use for hormonal imbalances with reports on an indirect loss of body weight, no direct links to adipocyte modulation have been described so far [13]. The data

of the clinical study using this plant extract presented here indicate slight beneficial effects by monk's pepper extract on skin properties. Based on the proposed role of AMT in skin aging, additional clinical studies to further assess the potential anti-aging effect of monk's pepper extract would be interesting. In addition, it would be worthwhile to test the clinical efficacy of plant extract B, as it demonstrated a similar effect on AMT modulation in the in vitro assay.

5. Conclusion

In this study, a new in vitro model for the transition of dermal adipocytes to myofibroblasts was developed. As the inhibition of AMT can potentially prevent from the negative consequences of AMT on the skin, the model allows to screen for new active cosmetic ingredients to counteract these effects. Using this model, it was possible to identify a plant extract based on monk's pepper which can modulate AMT and has been shown to have beneficial effects on the skin.

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7. References

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