TGFB Treated Placenta Derived Mesenchymal Stem Cells Selectively Promote Anti-Adipogenesis in Thyroid-Associated Ophthalmopathy

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Research Article

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Abstract

Background

Thyroid-associated ophthalmopathy (TAO) includes several ocular manifestations of the systemic autoimmune process, Graves’ disease. The key pathogenic cells are orbital fibroblasts (OFs). The OFs of most TAO patients are differentiated from pre-adipocytes to mature adipocytes, increased lipid and fat expansion are characteristic of ophthalmic disease. Human placental mesenchymal stem cells (hPMSCs) immunomodulate the pathogenesis. We previous showed that hPMSCs were therapeutic. Here, we prepared transforming growth factor β (TGFβ) treated hPMSCs (TGFβ-hPMSCs) to enhance adipogenesis inhibition in vitro and TAO mice.

Methods

To investigate anti-adipogenic effects in vitro, primary OFs were grown in differentiation medium for 2, 4, and 10 days. After co-cultur with hPMSCs and TGFβ-hPMSCs, the OFs were analyzed via quantitative real-time polymerase chain reaction, Oil red O staining and western blotting. Human PMSCs and TGFβ-hPMSCs were injected into the left orbits of TAO mice. We explored the anti-adipogenic pathway in play using SB431542, a TGFβ receptor kinase inhibitor.

Results

TGFβ-hPMSCs suppressed transcription of the adipogenic, lipogenic, and fibrotic genes PPARγ, C/EBPa, LEPTIN, AP2, SREBP2, HMGCR, α-SMA, FIBRONECTIN and IL-17 more so than did hPMSCs when adipogenesis-induced primary TAO OFs. Moreover, in TAO mice injected with TGFβ-hPMSCs, the adipose area was reduced compared to that after injection, of hPMSCs or a steroid, and the expression levels of the Ppar and C/ebp were more effectively suppressed. These anti-adipogenic effects were mediated by the SMAD 2 and SMAD3 pathways both in vitro and in vivo. TGFβ-hPMSCs inhibited inflammation as effectively as did a steroid both in vitro and in vivo, and reduced in vitro fibrosis.

Conclusion

TGFβ-hPMSCs reduced adipogenesis and lipogenesis in in vitro and TAO mice with experimental indicating that TGFβ-hPMSCs could serve as cellular therapy for TAO patients. Furthermore, TGFβ-hPMSCs exhibited anti-inflammatory and anti-fibrotic functions and thus, could be used to treat muscle fibrosis in TAO patients.

Introduction
Thyroid-associated ophthalmopathy (TAO) frequently manifests with thyroid dysfunction and is also known as thyroid eye disease or Graves’ ophthalmopathy (GO) [1]. Thyroid eye manifestations include proptosis, eyelid retraction, exposure keratopathy, restrictive strabismus, limitation of eye movement, compressive optic neuropathy, disfigured appearance and increases in the orbital fat, connective tissue and extraocular muscle volumes [2]. Orbital fibroblasts (OFs) in most TAO patients differentiate from pre-adipocytes into mature adipocytes, and thus express high levels of lipid and fat [3].

Human placental mesenchymal stem cells (hPMSCs) exhibit multilineage differentiation and potent immunomodulatory capacities useful in terms of tissue repair and in regenerative medicine [4]. Human PMSCs express high levels of human leukocyte antigen (HLA)-ABC and HLA-G [5]. Furthermore, phosphatase of the regenerating liver-1 (PRL-1) is overexpressed in hPMSCs, which are positive for CD13, CD90, CD105, and major histocompatibility complex (MHC) class I antigens including HLA-ABC and HLA-G but negative for MHC class II antigens including HLA-DR [6]. We previously showed that adipogenesis was inhibited by SREBP2-HMGCR signaling when PRL-1 was overexposed in hPMSCs cultured with GO fibroblasts [7].

Member of transforming growth factor β (TGFβ) superfamily regulate many cellular processes including apoptosis, inflammation, fibrosis and adipocyte differentiation [8]. TGFβ blocks adipocyte differentiation in vitro, and transgenic overexpression of TGFβ in adipose tissue also inhibits differentiation. TGFβ significantly reduces adipogenesis differentiation via TGFβ/SMAD signaling [9]. However, no study has yet explored whether TGFβ stimulated hPMSCs effect adipogenesis differentiation in TAO.

In this study, we investigated the inhibition effect on adipogenic differentiation from functionally enhanced hPMSCs using TGFβ in TAO OFs using in vitro and in vivo tests.

## Materials And Methods

### Cell preparation of hPMSCs and Orbital fibroblast

The orbital fibroblast isolation protocol was approved by the Institutional Review Committee (IRB-2018-01-007) of Bundang Cha Hospital in Seongnam, Korea, and all patients’ consent was obtained. Ophthalmic adipose tissue descriptions were obtained from patients with TAO during intraocular adipose decompression and controlled individuals without TAO history during orbital plastic surgery. The tissue was chopped and treated with collagenase (0.25 mg/mL; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 1 hour in shaking incubator. After incubation, the digested tissues were placed directly in culture dishes with DMEM/F12 containing 20% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Experiments were performed using fifth to eighth cell passage. Human placenta stem cells preparation and culturing were conducted as previously reported [10]. Human PMSCs were cultured in α-modified minimal essential medium (α-MEM; HyClone, Logan, UT, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 1% P/S (Thermo Fisher Scientific), 1 µg/mL...
heparin (Sigma-Aldrich, St. Louis, MO, USA), and 25 ng/mL human fibroblast growth factor-4 (hFGF-4; Peprotech, Rocky Hill, NJ, USA).

**Adipocyte differentiation**

Using a six-well plate, normal and TAO-derived OFs (4 × 10⁴/cm²) were seeded per well and incubated in DMEM medium supplemented with 33 µM biotin, 17 µM pantothenic acid, 0.2 nM triiodothyronine (T₃), 10 µg/mL transferrin, 0.2 µM carbaprostacyclin (cPGI₂; Cayman Chemical, Ann Arbor, MI, USA), 0.1 mM isobutylmethylxanthine (IBMX), 1 µM dexamethasone, and 1 µM insulin (all form Sigma-Aldrich). During initial differentiation stage, the incubation medium was replaced daily until 4 days. For maturation of adipocytes, the cells were incubated in a differentiation medium, without 1 µM dexamethasone, 0.1 mM IBMX and 1 µM insulin (all from Sigma-Aldrich). Cells were harvested at 2, 4 and 10 days for experiment.

**Co-culture experiments**

Before co-culturing, hPMSCs was treated recombinant human TGFβ1 at 20 ng/mL (Peprotech) for 24 h, and then adipose induced normal and TAO-derived OFs were co-cultured with naïve hPMSCs (OF + hPMSCs) or TGFβ-hPMSCs (OF + TGFβ-hPMSCs) using Transwell inserts (8 µm pore size; Corning, NY, USA) for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. In addition, OFs were pretreated with 10µM of TGFβ receptor kinase inhibitor, SB431542, (Selleckchem, Houston, TX, USA) right before co-culture with hPMSCs (SB + OF + hPMSCs) or TGFβ-hPMSCs ( SB + OF + TGFβ-hPMSCs). Cells were harvested after 24 h.

**MTT assay**

The hPMSCs were seeded at a density of 1×10⁴ cells/well in 96 well plates. After TGFβ treatment, the cells were incubated with an MTT solution (MTT: 2 mg/ml, Sigma-Aldrich) for 2 h at 37°C. The supernatants were aspirated, and DMSO (Sigma-Aldrich) were added to each well. After incubation for 15 minutes, absorbance was measured at 450 nm.

**Oil Red O staining**

Oil Red O staining was performed on 10th day of differentiation. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% formalin for 30 minutes, and cells were stained with Oil-Red O for 1 hour, were washed twice with water. After Oil Red O staining, cells were dissolved in isopropanol and quantified by measuring absorbance at 470 nm, and were photographed by a phase-contrast microscope (Olympus CKX41) at 20 × magnification.

**Quantitative real-time polymerase chain reaction**

Using TRlzl reagent (Ambion, Carlsbad, CA, USA), we isolated RNA from human OFs for cDNA synthesis. According to manufactor protocol, we synthesized cDNA using 1 ug of RNA. Gene expression was quantified with amfiSure qGreen Q-PCR Master Mix, Low ROX (GenDEPOT, Katy, TX, USA) and calculated by the delta delta CT method, and real-time PCR reactions were performed using a QuantStudio™1 Real-
Time PCR Instrument (Applied Biosystems, Foster City, CA, USA). The sequences of used primers are presented in Table 1.
Table 1

Human primer sequences using quantitative real time polymerase chain reaction

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**Development of an experimental mouse model of TAO using female BALB/c mice**

We generated a TAO disease model by injection pTriEx1.1Neo-hTSHR A-subunit plasmid to leg muscle using electroporation and characterized TAO disease model in our previous report [11]. The animals undergoing experimental TAO were divided into four groups: a treatment group injected with hPMSCs (3 × 10^5 cells/30 µL), a treatment group injected with TGFβ-hPMSCs (3 × 10^5 cells/30 µL), a treatment group injected with steroids (0.4 mg/each, triamcinolone acetonide, Dongkwang Pharmaceutical Co., Hanmi, South Korea), and a sham group (30 µL BSS PLUS). Intra-orbital injection was performed on the left orbit. One week after hPMSCs injection, the animals were sacrificed, after which orbital tissue was excised for histopathological analyses.

**Orbital tissue histopathology**
To quantify adipose area around the optic nerve, it was conducted using ZEISS Axio Scan. Z1 slide scanner (Carl Zeiss, Jena, Germany). The cross-sectional area of the orbital fat was normalized to the other-side adipose tissue area of each mouse. The adipose areas of the orbital sections of each mouse were evaluated in every group.

**Western blot analysis**

Protein lysates were prepared from animal orbital tissues and human OFs, orbital tissues from each group were homogenized with PRO-PREP solution (Intron, Gyeonggido, Korea). A total of 20 ug proteins were decomposed into sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and transferred to the membrane. The membranes transferred from gels, were incubated with primary antibodies such as anti-PPARγ (GeneTex, Irvine, CA, USA), C/EBPα (GeneTex), IGF-1R (GeneTex), p-SMAD3 (GeneTex), p-SMAD2 (GeneTex), IL-6 (GeneTex), TNFα (GeneTex), ICAM-1 (Thermo Fisher Scientific), TGFβ1 (GeneTex) and β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After washing, second antibodies (GeneTex), horseradish peroxidase-conjugated anti-rabbit or mouse IgG were diluted 1:5000 and were incubated with membranes at room temperature for 2 hours. The target protein bands were detected with enhanced chemiluminescence (ECL) solution (Bio-Rad Laboratories, Hercules, CA, USA) using an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK).

**Statistical analyses**

Data analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Significant differences were identified using t-test or nonparametric statistical test, followed by a Mann–Whitney U-test at a 5% significance level.

**Results**

**Characterization of hPMSCs treated with TGFβ1**

To investigate the effects of TGFβ1 on hPMSCs, hPMSCs were incubated with 20 ng/mL of TGFβ1 for 24 h. Cell viability of hPMSCs decreased by 25.9% (Fig. 1A). Next, we examined mRNA expressions of adipogenesis, lipogenesis and inflammation-related gene in hPMSCs treated with TGFβ1. As a result, adipogenesis marker genes, PPARγ, C/EBPα, LEPTIN, and HMGCR expressions were significantly decreased by TGFβ1. However, mRNA expressions of lipogenesis marker genes, IGF-1, IGF-1R, and INSIG2 were increased by TGFβ1 treatment (Fig. 1B). The expression of inflammation marker genes, TGFβ1, TGFβ2 and TNFα mRNA levels were also increased, while IL6, IL8, ICAM-1 and IL-1β expressions were decreased by TGFβ1 treatment (Fig. 1C). These results indicated that TGFβ could strongly enhance hPMSCs in anti-adipogenesis and anti-inflammatory effects.

**Effect of TGFβ-hPMSCs on TAO-derived OFs**

We confirmed changes in the levels of adipogenesis marker genes in OFs of TAO patients. PPARγ, C/EBPα, LEPTIN, and AP2 expression increased after the induction of adipogenesis and decreased on co-
culture with hPMSCs. The reductions were greater on co-culture with TGFβ-hPMSCs than with hPMSCs. The hPMSCs or TGFβ-hPMSCs co-culture were performed after the induction of OFs adipogenesis by SB431542 (TGF-β RI kinase Inhibitor) (Fig. 2A). The extents of differentiation of normal and TAO OFs were examined via Oil Red O staining. Both adipogenic-induced normal and TAO OFs exhibited significantly more lipid accumulation compared to the negative control. After co-culture with hPMSCs and TGFβ-hPMSCs lipid accumulation in TAO OFs was inhibited by 65.8% and 70.8% respectively, but not when the TAO OFs were pre-treated with SB431542 (Fig. 2B). TAO OFs co-cultured with hPMSCs also regulated mRNA expressions of lipogenic genes including INSIG1, INSIG2, SREBP2, HMGCR and IGFBP3 (Fig. 2C). In adipogenic TAO OFs, hPMSCs and TGFβ-hPMSCs increased the levels of mRNA expression of INSIG1, and hPMSCs was more effective than TGFβ-hPMSCs. INSIG1 and INSIG2 play roles in cholesterol homeostasis and precursor adipocyte differentiation [12]. Thus, both hPMSCs and TGFβ-hPMSCs regulated adipocyte differentiation. In adipogenic TAO OFs, the levels of mRNA of SREBP2, HMGCR and IGFBP3 were significantly increased compared to those of non-adipogenic TAO OFs. TGFβ-hPMSCs inhibited the expressions of these genes more so than did naïve hPMSCs (Fig. 2C). To investigate whether hPMSCs is involved in other reaction, we confirmed mRNA expressions of pro-fibrotic genes including α-SMA and FIBRONECTIN, and a gene related to inflammation, IL-17. The expression levels were increased in adipogenic TAO OFs, and co-culture with TGFβ-hPMSCs inhibited expression more so than did co-culture with naïve hPMSCs. We used SB431542 to confirm the TGFβ-mediated anti-adipogenic effects of TGFβ-hPMSCs (Fig. 2D). Thus, hPMSCs inhibited adipogenesis, lipogenesis, fibrosis and inflammation in adipogenic TAO OFs, and the inhibitory effects of TGFβ-hPMSCs were greater than those of hPMSCs.

Pathology assessment

We assessed the pathologies of TAO groups and analyzed adipogenesis and inflammation-related target protein expressions in orbital tissues of TAO animals. We measured adipose area around the optic nerve. The average of TAO animals (Sham) was significantly larger by 206.5% than the negative control animals (NC). Injection of hPMSCs or a steroid (STE) significantly decreased adipose area by 72.9% and 72.3%, respectively. Particularly, TGFβ-hPMSCs injection significantly more inhibited the adipose area by 79.6% (Fig. 3A).

TGFβ-hPMSCs inhibit adipogenesis and inflammation in the TAO animals

We next examined levels of the adipogenic proteins Igf-1r, Pparγ, and C/ebpa. Pparγ levels were significantly reduced by hPMSCs and TGFβ-hPMSCs reduced the Igf-1r, Pparγ and C/ebpa levels in the TAO animals (Fig. 3B). We then examined levels of the inflammatory proteins Icam-1, Tgfβ1, Il6, and Tnfa protein. TGFβ-hPMSCs more effectively inhibited Icam-1 and Tgfβ1 by 57.9% and 57.4%, respectively, compared to 45.2% and 45.1% by hPMSCs (Fig. 3C).

TGFβ-hPMSCs regulate TAO OFs via the SMAD pathway.
To investigate mediated signaling in TGFβ-hPMSCs induced regulation, we examined mRNA expression of SMAD family. We analyzed the phosphorylation of SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) in TAO OFs. Compared to negative control, p-SMAD2 and p-SMAD3 levels decreased in adipogenic TAO OFs and hPMSCs co-cultured. However, TGFβ-hPMSCs significantly increased p-SMAD2 by 137.8%, and p-SMAD3 by 106.1% than adipogenic TAO OFs (Fig. 4A). Additionally, we analyzed the p-Smad2 and p-Smad3 protein levels in TAO animals (Fig. 4B). The p-Smad3 level was not regulated by treatments in TAO animals. Human PMSCs significantly reduced p-Smad2 than sham group, while TGFβ-hPMSCs significantly increased p-Smad2 expression by 161.3% (Fig. 4B). From these results, we could assume that TGFβ-hPMSCs inhibited activities of adipogenic, lipogenic, and fibrotic genes through SMAD2/3 pathway (Fig. 5).

Discussion

TGFβ profoundly affects the differentiation of many cell types of mesenchymal origin, including pre-adipocytes, osteoblasts, and myoblasts [13]. Members of the TGFβ family regulate MSC lineage selection and progression of mesenchymal differentiation to specific cell, types by controlling the expression and activity of key transcription factors [14]. TGFβ family members, including BMPs, TGF-βs, activins, and inhibins regulate the differentiation of early bone marrow stromal cells (BMSCs) into the mature matrix-secreting osteoblasts and osteocytes required for normal late myoblast differentiation, potentially via Smad-independent mechanisms, a sub-signal of TGFβ [15]. In addition, many studies have been reported that inflammation and adipogenesis are regulated by TGFβ, which inhibits adipogenesis in unloaded bone marrow stromal cells [16] and also, inhibits adipocyte differentiation by Smad3 interacts with the CCAAT/Enhancer-binding Protein (C/EBP) [17]. Latent TGFβ1 protects against renal fibrosis and inflammation [18, 19] and suppresses T-cell proliferation and activation through Treg differentiation [20].

Undifferentiated MSCs initially differentiate into a pre-adipocyte stage and then proliferate by mitotic clonal expansion. Hormonal cues stimulate further differentiation by initiating production of C/EBPs β and δ, encoded by Cebpb and Cebpd, respectively. These transcription factors activate PPARγ and C/EBPa leading to growth arrest for terminal differentiation and the expression of adipocyte genes including FABP4/AP2 and LEPTIN [21]. The time course of adipogenesis differentiation varied in earlier reports, and our time course of genes expressed in this study was also different. INSIG2 was more highly expressed in hPMSCs than TGFβ-hPMSCs but this was not associated with any change in the extent of adipogenesis. We recently showed that hPMSCs inhibited TAO OFs derived adipogenesis via IGFBP when PRL-1 was overexpressed [7]. Although various orbital pathologies have been examined, studies the therapeutic effects of stem cells have been insufficient. We found that TGFβ enhanced the anti-adipogenic effects of hPMSCs on TAO both in vitro and in vivo. TGFβ-hPMSCs decreased IGFBP3 expression, indicating that adipogenesis was inhibited via signals other than IGFBPs. The powerful anti-adipogenesis effect of TGFβ-hPMSCs was mediated via SMAD signaling.

The SMAD family of transcription factors interact with TGFβ receptors to propagate signals. The receptors two transmembrane protein serine/threonine kinases; receptor types I and II, are brought
together by the ligand, which acts as a receptor assembly factor. Type I receptors specifically recognize receptor-activated SMADs (R-SMADs), these include SMAD2/3, which are also recognized by the TGFβ and activin receptors, and SMAD1/5/8, recognized by BMP receptors [22]. TGFβ binding activates SMAD2/3, and suppresses pre-adipocyte differentiation. However, BMP-like ligands that primarily activate the SMAD1/5/8 pathways, increase adipocyte numbers [23]. Activated SMAD2/3 inhibits adipogenesis, this is rescued by addition of SMAD2/3 upstream SB431542 [19, 24, 25].

Furthermore, the role of TGFβ in inflammatory diseases is defective TGFβ associated SMAD pathway because of high SMAD7, the protein binds to TGFβRI and prevents TGFβ-induced SMAD2/3 phosphorylation, thus amplifying the expression of inflammatory genes in inflammatory bowel disease [26]. TGF-β inhibits inflammation in combination with p-SMAD2/3, but SMAD7 blocks this increasing inflammation in children with environmental enteropathy [27]. We found, in vivo that the anti-inflammatory effects of TGFβ-hPMSCs were as good as those of a steroid, and better than those of hPMSCs. Thus, TGFβ-hPMSCs may be used to treat both inflammatory diseases and TAO. In this study, hPMSCs alone inhibited adipogenesis, but TGFβ enhanced such inhibition. We previously showed that hPMSCs have immune modulatory effects, inhibiting adipogenesis via anti-inflammatory effects [11]. TGFβ induced SMAD2/3 signaling is anti-inflammation conditions, therefore they could provide the adipogenesis inhibitory function. Phosphorylation of TGFβ induced SMAD2/3 decreased in TAO OFs treated with the TGFβ inhibitor SB431542.

In addition, TGFβ plays a key role in fibrosis [17, 28, 29]. Fibroblasts are activated by TGFβ to transition into myofibroblasts, a key effector cells. Myofibroblasts are characterized by high levels of contractile proteins including α-smooth muscle actin (α-SMA), collagens I, III, V and fibronectin [30, 31]. However, we found that when hPMSCs treated with TGFβ were co-cultured with TAO OFs, the α-SMA and fibronectin levels fell. We expected that TAO OFs fibrosis would be aggravated on co-culture with TGFβ-hPMSCs because adipogenesis was induced. However, we observed the reverse. CD4+ T helper cells are involved in renal inflammation and fibrosis and it has been reported that hPMSCs can convert an inflammatory environment into an anti-inflammatory environment by affecting the polarization of CD4+ T cells, and macrophages [32]. Therefore, even when fibrosis in stimulated, TAO OFs are expected to suppress fibrosis due to the influence of hPMSCs rather than that of TGFβ. TGFβ-hPMSCs did not stimulate fibrosis of TAO OFs but rather, inhibited the differentiation into the myoblasts as well as adipogenesis. Further researches on the roles played by TGFβ-hPMSCs in TAO fibrosis are needed.

**Conclusion**

We found that TGFβ-hPMSCs selectively enhanced the anti-adipogenic effects both in vitro and in vivo of TAO compared with naïve hPMSCs. These results show that TGFβ-hPMSCs activated SMAD2/3, and phosphorylated SMAD2/3 which suppressed transcriptions of lipogenic genes (e.g. HMGCR and SREBP2) and adipogenic genes (e.g. PPARγ, C/EBPa, Leptin and aP2) as well as fibrotic gene, including α-SMA and Fibronectin. In conclusion this study provides a new and safe method to enhance the anti-adipogenic function of hPMSCs that can be used to treat TAO patients.
Abbreviations

TAO: Thyroid-associated ophthalmopathy; OFs: Orbital fibroblasts; hPMSCs: Human placental mesenchymal stem cells; TGFβ: Transforming growth factor β

Declarations

Ethics approval and consent to participate

The process of obtaining orbital adipose tissue was approved by the Institutional Review Board of Bundang CHA Medical Center (Seongnam-si, South Korea) and the animal protocol was approved by the Institutional Animal Care and Use Committee of CHA Bundang Medical Center. All patients consented to the proper use for research.

Consent for publication

Not applicable.

Availability of data and materials

All data and materials are available upon request.

Competing interests

The authors declare no conflicts of interest regarding the publication of this paper.

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Authors’ contributions

HS and MP contributed to the experiments and data analysis and manuscript writing. JPB kindly provided Plasmid DNA and consultants for construction of GO model. HL contributed conception and design, manuscript writing and final approval of the manuscript.

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References


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Figures

Figure 1

Characterization of hPMSCs with TGFβ treatment. (A) Cell viability was determined by MTT assay at 24 h after TGFβ (20ng/mL) treatment. (B) The relative mRNA expression of adipogenesis and lipogenesis marker genes (e.g. *PPARγ, C/EBPα, LEPTIN, SREBP2, HMGCR, INSIG1, INSIG2, IGF-1, and IGF-1R*) were analyzed by qRT-PCR after TGFβ treatment. (C) The relative mRNA expression of inflammation marker genes (e.g. *TGFβ1, TGFβ2, TNFα, IL-6, IL-8, ICAM-1*, and *IL-1β*) were analyzed by qRT-PCR on hour 24 after TGFβ treatment (*p<0.05, **p<0.01 vs. hPMSCs).

Figure 2

TGFβ-hPMSCs inhibit adipogenesis, lipogenesis and fibrosis in TAO-derived OFs. (A) The mRNA expression of adipogenesis marker genes (e.g. *PPARγ, C/EBPα, LEPTIN, and AP2*) were analyzed by qRT-PCR. (B) Quantification of ORO staining in differentiated OFs. The graph shows relative quantification at 470 nm absorbance. Data was presented as a fold change (means ± SEM). (C) The mRNA expression of lipogenesis genes (e.g. *IGFBP3, INSIG1, INSIG2, SREBP2*, and *HMGCR*). (D) Fibrosis and inflammation genes (e.g. *α-SMA, FIBRONECTIN, and IL17*) were analyzed by qRT-PCR. Significantly different values between the groups are indicated with marks (*p<0.05, **p<0.01, ***p<0.001 Negative control vs. adipogenesis induced OFs (-); #p < 0.05, ##p<0.01, ###p<0.001 vs. adipogenesis induced OFs (-); +p<0.05, ++p<0.01, +++<0.001 vs. OF+hPMSCs; †p<0.05, ††p<0.01, †††<0.001 vs. OF+TGFβ-hPMSCs; ‡‡‡p<0.001 vs.SB+OF+hPMSCs). The time dependent manner of genes expression were analyzed as follows; day 2 (*PPARγ*); day 4 (*C/EBPα, LEPTIN, SREBP2, HMGCR*, and *IL-17*); day 10 (*AP2, IGFBP3, INSIG1, INSIG2, α-SMA*, and *FIBRONECTIN*).

Figure 3

Histologic analysis of TAO animals treated with hPMSCs, TGFβ-hPMSCs and steroid injection. (A) H&E-stained section from TAO mice with an expansion of adipose area around the optic nerve (Magnification, x100). Data was presented as a fold change (means ± SEM) of adipose area volume around optic nerve compared with the sham (*p<0.05 Negative control vs. Sham; #p<0.05 vs. Sham). Negative control n=3, Sham n=4, hPMSCs n=3, TGFβ-hPMSCs n=3, STE (steroid) n=3. By western blotting, (B) adipogenesis
related-protein (e.g. Igf-1r, Pparγ and C/ebpα) expressions of orbital tissues were analyzed. Sham n=3, hPMSCs n=3, TGFβ-hPMSCs n=4, STE (steroid) n=2. (C) inflammation related-protein (e.g. Icam-1, Tgfβ1, Il-6 and Tnfα) expressions of orbital tissues were analyzed. Sham n=3, hPMSCs n=3, TGFβ-hPMSCs n=2, STE (steroid) n=2. Data was presented as the fold changes (means ± SEM) Expression levels were normalized to β-actin and the values of OS were divided OD (*p<0.05 Negative control vs. Sham; #p<0.05, ##p<0.01, ###p<0.001 vs. Sham; †p<0.05 vs. TGFβ-hPMSCs). OD, oculus dexter; OS, oculus sinister.

Figure 4

**TGFβ-hPMSCs co-culture regulated SMAD signaling pathway.** (A) Protein lysates from TAO OFs were used to western blot for analysis of Phosphorylation-SMAD protein on day 10 (e.g. p-SMAD2 and p-SMAD3) expression levels (***p<0.001 vs. Negative control vs. adipogenesis induced OFs; #p<0.05, ##p<0.01 vs. adipogenesis induced OFs; +p<0.05, ++p<0.01 vs. OF+hPMSCs; +++<0.001 vs. OF+TGFβ-hPMSCs; †p<0.05 vs. SB+OF+ hPMSCs). (B) Mice orbital tissue lysates were investigated by western blot to detect p-Smad2 and p-Smad3 expression levels. Expression levels were normalized to β-actin and the values of OS were divided OD. Data was presented as a fold change (means ± SEM). (#p<0.05 vs. Sham; ++p<0.01, +++p<0.001 vs. hPMSCs). Sham n=3, hPMSCs n=3, TGFβ-hPMSCs n=2, STE (steroid) n=3.

Figure 5

**Proposed pathway of TGFβ-hPMSCs regarding anti-adipogenesis in TAO.**

TGFβ-hPMSCs stimulate TGFβ signal transduction by binding to transmembrane receptors. The phosphorylated receptors transmit signaling by phosphorylating Smad 2/3 and inducing their binding to Smad 4. The Smad complex translocate into the nucleus and action as transcription factors to inhibit lipogenic genes (e.g. HMGCR and SREBP2), adipogenic genes (e.g. PPARγ, C/EBPα, Leptin and aP2) transcription as well as fibrotic genes transcription, including a-SMA and Fibronectin.