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Original article

Transcriptional pattern of TGF- β 1 inhibitory effect on mouse C2C12 myoblasts differentiation

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Abstract

The aim of the present study was to define the effect of TGF- β 1 on C2C12 myoblasts myogenesis. TGF- β 1 together with its receptor is a negative auto-paracrine regulator of myogenesis, which influences the proliferation, differentiation, and functions of muscle cells. TGF- β 1 exerts highly significant inhibitory effect on differentiation of C2C12 mouse myoblasts manifested by the impairment of cell fusion and very low expression of myosin heavy chain. The study of differentiating C2C12 mouse myoblasts treated with TGF- β 1 revealed 502 genes (436 down-regulated and 66 up-regulated) with statistically different expression. TGF- β 1-regulated genes were identified to be involved in 29 biological processes, 29 molecular functions groups and 59 pathways. The strongest inhibiting effect of TGF- β 1 was observed in the cadherin and Wnt pathways. The key-genes that could play the role of TGF- β 1 targets during myoblasts differentiation was identified such as: *Max*, *Creb1*, *Ccna2*, *Bax*, *Mdf1*, *Tef*, *Tubg1*, *Cxcl5*, *Rho*, *Calca* and *Lgals4*.

Key words: C2C12 myoblasts, differentiation, DNA microarrays, myogenesis, TGF- β 1

Introduction

Transforming growth factor- β 1 (TGF- β 1) belongs to the transforming growth factor beta super-family, a large group of proteins which play a significant role in regulation of cell growth and differentiation. TGF- β 1 was described in 1983, as a first factor belonging to this family (Miyazono et al. 1988). TGF- β 1 is a 25 kDa homodimeric protein consisting of two subunits, each with a molecular weight 12.5 kDa and 112 amino-acids joined by disulphide bounds. TGF- β 1 is synthesized as a 110 kDa propeptide containing short hydrophobic signaling peptide, latency associated peptide (LAP) and an active TGF- β 1 domain in C-terminal region (Klass et al. 2009). TGF- β 1 is an

inhibitor of myogenic cell proliferation and differentiation. Results presented by Zimowska et al. (2009) indicate that the control of TGF- β 1 activity is important to improve regeneration of injured muscle and accelerate myoblast differentiation, in part through changes in glycosaminoglycans composition of muscle cell environment. TGF- β 1 can also inhibit mitosis in epithelial and epidermal cells, blood precursor cells, liver, ovary, lymphatic and endothelial cells. The role of TGF- β 1 is also the regulation of angiogenesis and simulation of extracellular matrix components synthesis. TGF- β 1 is a subject of intense investigations aiming to evaluate its role in pathogenesis of different diseases, especially tumors (Wrighton et al. 2009). The signal transduction of all proteins belonging to

TGF- β superfamily, including TGF- β 1, occurs through two types of trans-membrane receptors with serine-threonine kinase activity. TGF- β 1 initiates downstream signals by binding directly to type II receptor (RII) and activation of type I receptor (RI) (Le Grand et al. 2007). Afterwards the presence of Smad2/Smad3 dimers (R-Smads) enables phosphorylation of RII and RI receptor complex and formation of a heterodimer with Smad4. The R-Smad/Smad4 complex is then translocated into the nucleus where it binds with one of DNA-binding proteins which serve as activators or repressors of target gene transcription (Moustakas 2002).

TGF- β 1 modulates myoblasts activity by inhibition of their proliferation and differentiation, which was shown *in vitro* in C2C12, BC3H1 and L6 cell lines (Olson 1992, Kollias et al. 2006). Inhibition of differentiation occurs by down-regulation of MyoD and myogenin expression. Moreover, it was shown that TGF- β 1 could directly influence myostatin (growth and differentiation factor 8 – GDF-8) expression (Budasz-Świdorska et al. 2005). The inhibition of myoblast proliferation by TGF- β 1 occurs through inhibition of Cdk4 expression at the translational level, leading to cell cycle arrest in the G1 phase. Inhibition of myoblast differentiation by TGF- β 1 occurs by Smad signaling pathway, which activates or suppresses target genes. Smad-3 binds to the *E-box* sequence of muscle regulatory genes and inhibits binding of bHLH proteins with this sequence (Klass et al. 2009). Apart of TGF- β 1 there are other factors which can suppress myoblast differentiation e.g. myostatin (GDF-8) (Budasz-Świdorska et al. 2005), glycogen synthase kinase 3 beta (GSK3 β) (Van der Valken et al. 2008) or inhibitors of differentiation (Id) (Clever et al. 2010).

So far the knowledge about genes negatively regulated by TGF- β 1 during myogenesis is still obscure. In the present study, DNA microarray method was applied to investigate a wide-spectrum of gene expression profiling to identify the potential genes which are responsible for inhibition of C2C12 myogenic cells differentiation by TGF- β 1.

Materials and Methods

Media and reagents

DMEM with Glutamax, phosphate buffered saline (PBS) [pH 7.4], fetal bovine serum (FBS), horse serum (HS) and antibiotics: penicillin-streptomycin, fungizone and gentamycin sulphate were purchased from Gibco BRL (UK). Primary monoclonal rabbit anti-mouse MyHC (H-300) antibody was delivered by Santa Cruz Biotechnology Inc. (USA). Alexa Fluor 488 chicken anti-rabbit IgG secondary antibody and

7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (Germany). Sterile conical flasks, and Lab-Teks were supplied by Nunc Inc. (USA). Sterile Petri dishes and disposable pipettes were purchased from Corning Glass Co. (USA).

Cell culture

The mouse skeletal muscle cell line C2C12 was purchased from European Collection of Animal Cell Culture (UK). Cells were cultured as described before (Budasz-Świdorska et al. 2005). Preliminary experiments were done to assess the most effective dose of TGF- β 1 and time of the experiment. 2 ng/ml TGF- β 1 supplementation of experimental medium and 6-day time duration have been chosen. Control medium did not contain TGF- β 1. After sixth day, cells were harvested for RNA isolation or fixed for staining for confocal microscopy.

Experimental procedures

For RNA isolation, C2C12 cells were cultured on Petri dishes in proliferation medium (10% FBS/DMEM/antibiotics) until 80% of confluence. The medium was then replaced with differentiation medium (2% HS/DMEM/antibiotics) for next 6 days. Pictures of each stage of differentiation were taken using contrast-phase microscopy. After sixth day, cells were harvested and stored at -80°C until analysis. For confocal microscopy cells were cultured on a 8-chamber Lab-Tek slides until they reached 80% confluence and then were differentiated with 2% HS/DMEM for 6 days. Afterwards, cells were fixed by dehydration. Five separate experiments were performed.

RNA isolation and validation, probe labelling, hybridization, signal detection and quantification were done as described before (Sadkowski et al. 2008, 2009). In this study Mouse Genome OpArray v 4.0.1 (Operon, Germany) slides were used.

Confocal Microscopy

The cells were fixed by washing in 0.25% paraformaldehyde and were incubated in ice-cold 70% methanol. Samples were stored at -80°C until staining. Then cells were incubated for 1 h with the primary antibody (anti-MyHC) diluted 1:250 with PBS and after triple washing were incubated 1 hour with Alexa Fluor 488 secondary antibody 1:500 (anti-rabbit) at 4°C in the dark. The cells were finally incubated for 30 min with 5 μ g/ml of 7-aminoactinomycin D (7-AAD) at room temperature in the dark to stain the DNA of nuclei. Preparations were stored in the dark at 4°C for

24h before analysis. Images from confocal microscope FV-500 (Olympus Optical, Germany) were analyzed using MicroImage software (Olympus, Germany). For each culture stage 15 pictures were taken.

Results

The influence of TGF- β 1 on differentiation of C2C12 myoblasts

TGF- β 1 exerted a strong inhibitory effect on differentiation of mouse C2C12 myoblasts. It was evident in contrast phase images of control cultures where myotubes were visible from 48 h of differentiation period. (Figure 1a, upper panel). Addition of TGF- β 1 (2 ng/ml) exerted an inhibitory effect on fusion of myoblasts and formation of myotubes during the 6-day observation period (Figure 1a, lower panel). Moreover, there was observed an increase in the number of necrotic cells in TGF- β 1-treated cultures. Inhibition of C2C12 myoblasts differentiation by TGF- β 1 was also shown by immunofluorescence analysis of myosin heavy chain expression in cultures on the sixth day of myoblasts differentiation. MyHC-related fluorescence was visible only in confocal images containing myotubes from control cultures (Figure 1b, upper panel). There was no MyHC expression in TGF- β 1-treated cultures (Figure 1b, lower panel). Integrated optical density analysis (IOD) was calculated using MicroImage software. There was a significantly ($P \leq 0.0001$) lower expression of MyHC in TGF- β 1-treated myoblasts in comparison with control cultures (Fig. 1c).

The influence of TGF- β 1 on transcriptomic profile of differentiating C2C12 myoblasts

Global potentiometric analysis of gene expression profile in TGF- β 1-treated C2C12 myoblasts was performed using the DNA microarray technique. Comparison of transcriptomic profiles between cells treated with TGF- β 1 and control cultures revealed 502 genes, which exhibited statistically significant differences in expression, with at least 1.6 fold change. TGF- β 1 treatment caused down-regulation of 436 genes and an up-regulation of 66 genes. The role of these particular genes was evaluated with *Panther* software, that allows to classify identified genes in respect to their molecular function, biological process and metabolic pathways.

Classification of identified genes according to their molecular functions revealed 29 groups of genes significantly affected by TGF- β 1 (Fig. 2). The majority of genes were associated with groups such as: nucleic

acid binding (65), receptors (51), transcription factors (38), transferase (30) and hydrolase (25).

Classification of genes according to biological processes revealed 29 processes in which genes significantly affected by TGF- β 1 were involved (Fig. 3). The highest numbers of genes were involved in: signal transduction (106), nucleoside, nucleotide and nucleic acid metabolism (81), developmental processes (64) and protein metabolism and modification (57). Analysis of the results according to *Pathway Architect* software (Stratagene USA) showed connections between the products of genes identified in this study and genes described in the literature. This allowed to present the network of reciprocal interactions between protein products of the investigated genes. In the network there were also proteins of putative special significance, which formed junctions converging with other pathways. The genes encoding these proteins play or could play an essential role in proliferation and the developmental processes. The key genes, with decreased expression under the influence of TGF- β 1 were: Max protein (*Max*), cAMP responsive element binding protein 1 (*Creb1*), cyclin A2 (*Ccna2*), Bcl2-associated X protein (*Bax*), MyoD family inhibitor (*Mdf1*), thyrotroph embryonic factor (*Tef*), tubulin gamma1 (*Tubg1*), chemokine (C-X-C motif) ligand 5 (*Cxcl5*), rhodopsin (*Rho*). Whereas key genes, with increased expression were: calcitonin/calcitonin-related polypeptide alpha (*Calca*) and lectin, galactose binding, soluble 4 (*Lgals4*) (Fig. 4).

Classification of genes according to signaling pathways revealed that 59 signaling pathways were affected by TGF- β 1. Analysis of the role of the identified genes in various pathways showed that the largest number of gene products were involved in the Wnt signaling pathway represented by 12 genes, cadherin signaling pathways represented by 10 genes (*Pcdhgb8*, *Wnt16*, *ErbB4*, *Pcdhgc4*, *Pcdhb16*, *Fat3*, *Dchs2*, *Pcdhb20*, *Pcdh1*, *Pcdhb17*), inflammation mediated by chemokine and cytokine signaling pathway represented by 7 genes (*Fpr11*, *Tyk2*, *Plce1*, *Alox12*, *Xcr1*, *Inpp11*, *Rgs4*), Huntington disease pathway represented by 7 genes (*Synj2*, *Arfp2*, *Grin2c*, *Dnahc8*, *AI427515*, *Dnahc17*, *Bax*), Notch signaling pathway represented by 5 genes (*Numb*, *Dll4*, *Heyl*, *Fut8*, *Ncstn*) and TGF-beta signaling pathway represented by 3 genes (*Dcp1a*, *Smad5*, *Foxp2*). To localize the products of gene expression of the metabolic pathways in skeletal muscle fibers *Pathway Architect* software was used. The result of this analysis was the network describing the relationship between the products of the identified genes belonging to the most represented pathways: Wnt and cadherin signaling pathway (Fig. 5). Among the genes of the Wnt signaling pathway 5 groups of genes were distinguished: alpha-catenin – 1 gene (*Cttnal1*); cadherin – 8 genes (*Pcdhb17*; *Pcdhgc4*; *Pcdhb16*; *Fat3*; *Dchs2*; *Pcdhb20*;

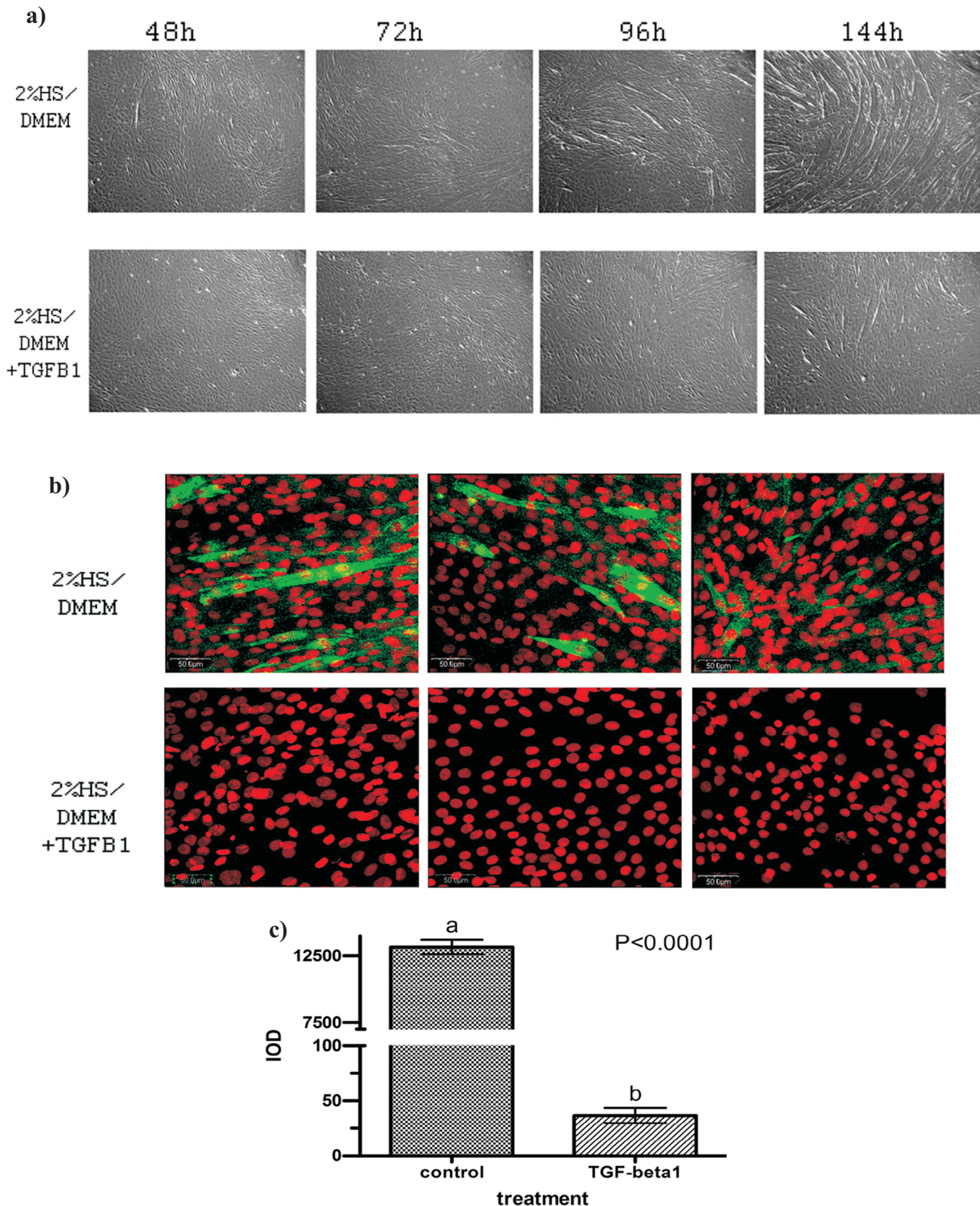


Fig. 1. a) Six days differentiation process of C2C12 myoblasts (100x magnification). Control culture stimulated to differentiation in DMEM/2% HS for 48, 72, 96 and 144 hours (upper panel). Experimental culture maintained in above medium supplemented with 2 ng/ml of TGF- β 1 (lower panel), b) Images from confocal microscopy showing expression of MyHC (Alexa 488 – green) and the DNA of nuclei (7-AAD – red) after six days of differentiation in control (upper panel) and TGF- β 1 treated culture (lower panel), c) Influence of TGF- β 1 on MyHC levels in myotubes of C2C12 mouse myoblasts. Results are showed as mean \pm SEM of MyHC integrated optical density (IOD). Means were calculated from 15 digital images for each sample. Values a and b indicate a significant statistical difference ($P \leq 0.0001$) between experimental culture treated with 2 ng/ml TGF- β 1 and control culture analyzed with *t*-Student test ($n = 5$).

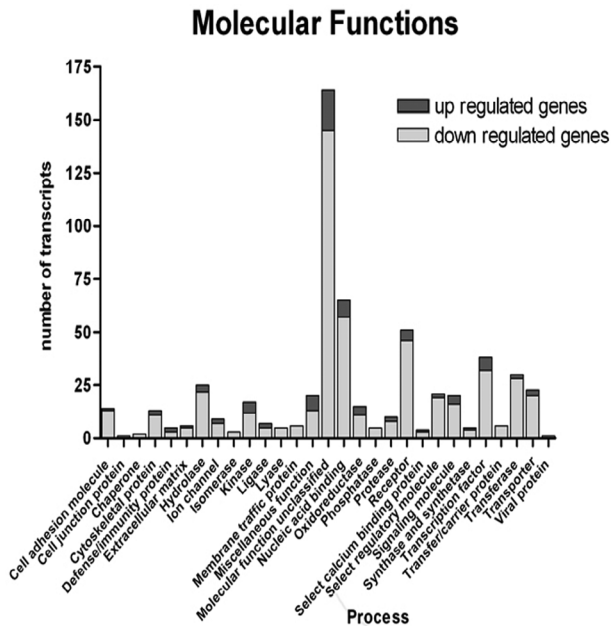


Fig. 2. Numbers of identified genes, which expression was changed in C2C12 cells under TGF- β 1 treatment, according to their molecular function. Based on *Panther* classification software ($n = 5$).

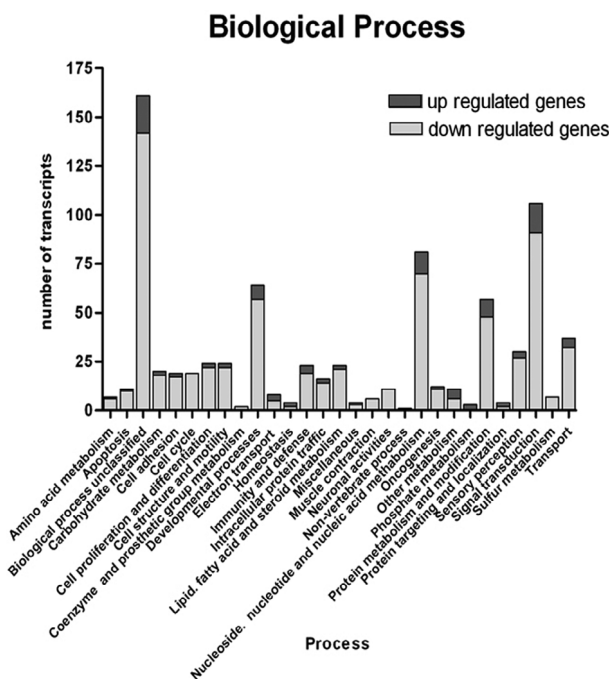


Fig. 3. Numbers of identified genes, which expression was changed in C2C12 cells under TGF- β 1 treatment, according to the biological process they were involved in. Based on *Panther* classification software ($n = 5$).

Pcdh1; *Pcdhgb8*); mothers against decapentaplegic homolog 4 – 1 gene (*Smad5*); Wingless-type MMTV integration site family member – 1 gene (*Wnt16*) and Wnt target genes – 1 gene (*Hoxc6*). In the case of

cadherin signaling pathway, only one gene was specific for this pathway (*ErbB4*), whereas remaining 9 genes were common with Wnt signaling pathway (*Pcdhb17*, *Pcdhgc4*, *Pcdhb16*, *Fat3*, *Dchs2*, *Pcdhb20*, *Pcdh1*, *Pcdhgb8*, *Wnt16*).

Discussion

This is the first transcriptomic study showing the effect of TGF- β 1 on global gene expression in mouse C2C12 myoblasts stimulated to differentiation. Inhibition of cell differentiation by TGF- β 1 (Fig. 1) was accompanied by a significant change in expression of hundreds of genes. The analyses of ontology of identified genes were performed using *Panther* and *Pathway Architect* software and genes with at least 1.6-fold change in expression were classified (Fig. 2 and 3). The change in their expression after TGF- β 1 treatment allowed to identify their indirect inhibitory influence on muscle growth and development. However, the interpretation of these results is challenging because of many of identified genes were not described earlier as genes involved in muscle differentiation.

Since the majority of 502 regulated genes were down-regulated (436 genes), we can hypothesize that TGF- β 1 shows mainly inhibitory effect on gene expression. Based on these results, we identified 11 key genes, which are particularly important because of their location at the intersection of many metabolic pathways (Fig. 4). Proteins encoded by assigned key genes are localized in different cell compartments and are involved in different molecular functions, but they create functional network, which regulates proliferation and differentiation of muscle cells.

Max (Max protein) is a small protein that could form homo- and heterodimers with the Mad, Mxi1 and Myc protein. Complexes of Myc-Max are known as exerting influence on global regulation of genes, enclosing: cell growth, entry into the cell cycle, proliferation and embryonic development through the binding with the *E-Box* sites which allows initiation of transcription. In our studies, TGF- β 1 caused decrease of *Max* gene expression by 1.83 fold change (Table 1, position 110. All of identified in this study genes are presented on the website: <http://www.sggw.info/knf/microarray/tgfb1/index.html>) This could lead to the inhibition of b/HLH/Z domain proteins signaling pathway and slow down the differentiation process.

Creb1 (cAMP-responsive element binding protein) is responsible for regulation of mRNA transcription under the influence of cAMP. It was found that Creb1 is engaged in protein kinase A signaling pathway during myogenesis induced by Wnt proteins (Chen et al. 2005). Data from the literature indicate that TGF- β 1 could reduce Creb phosphorylation,

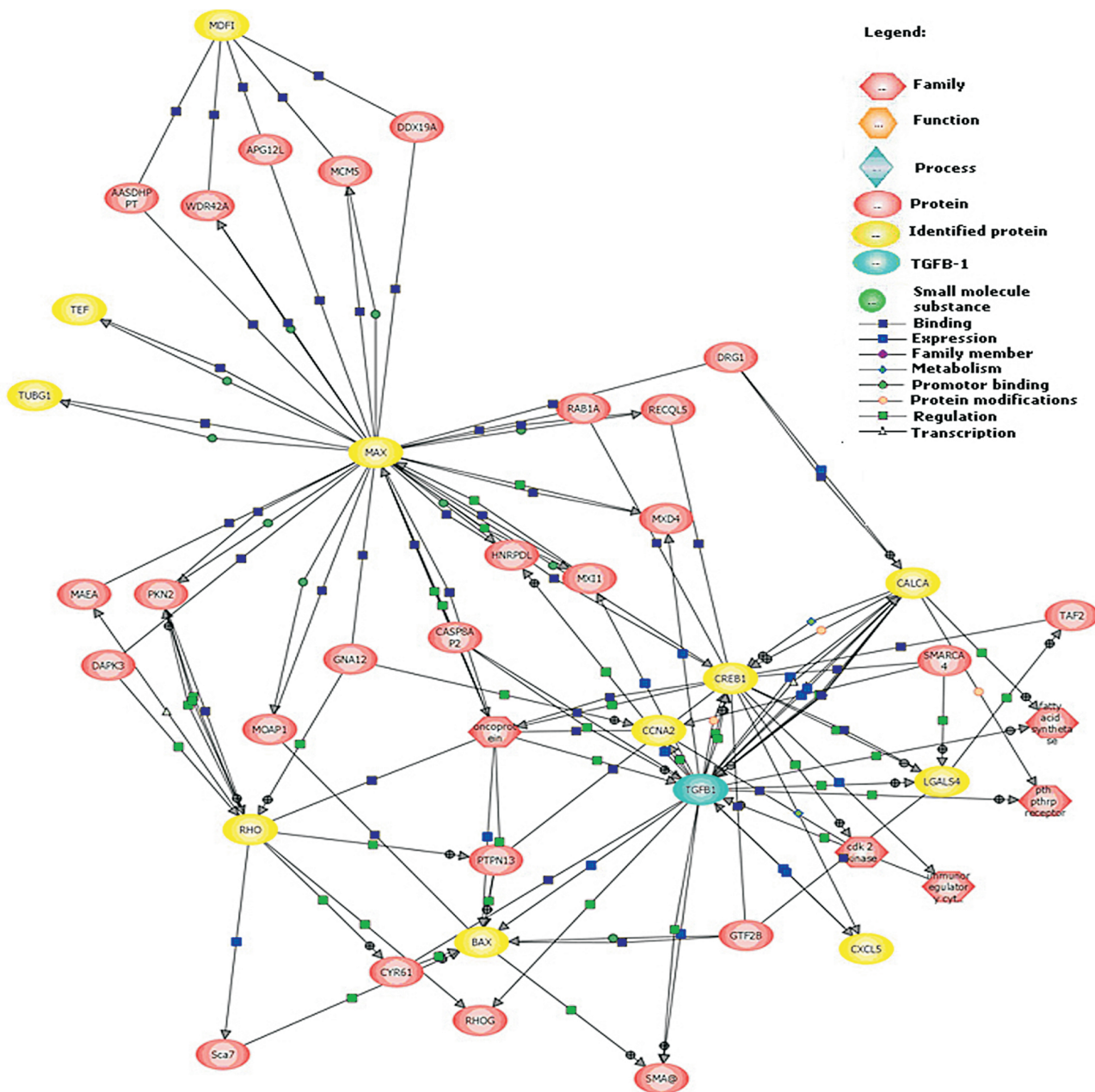


Fig. 4. Network of mutual interactions between TGF- β 1 (blue), products of identified key genes (yellow), and products of other genes described in the literature (red). The analysis was performed by using *Pathway Architect* software.

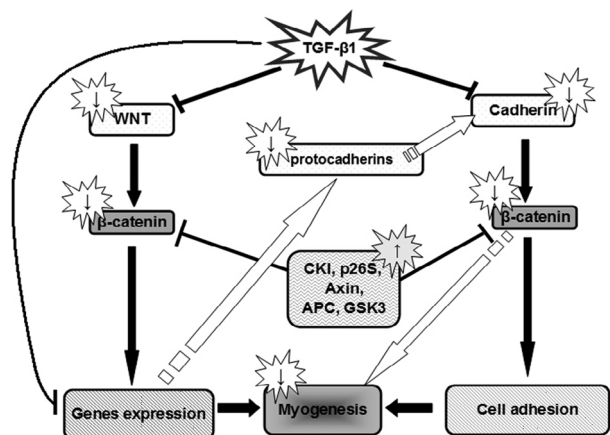


Fig. 5. Schematic illustration of supposed influence of TGF- β 1 on Wnt signalling pathway and cadherin signaling pathway through β -catenin and APC complex, leading to decrease of genes transcription and inhibition of myogenesis.

which results in lower activity of cyclin A promoter and inhibits the mitotic cycle. In our study, TGF- β 1 caused decrease of *Creb1* gene expression by 2.35 fold (Table 1, position 10), which probably reduced *Ccna* levels and as a result disturbed *MyoD* pathway inhibiting cell differentiation processes.

Ccna2 (cyclin A2) – is a regulatory component of cyclin-dependent kinase 2 (Cdk2), that plays an essential role in the S phase of cell cycle (G1 / S). *Ccna2* forms a complex with Cdk1 and enables the start of mitosis (G2 / M). Previous studies confirmed the inhibitory effect of TGF- β 1 on the expression of Cdk and *Ccna2* (Rama et al. 2003). Reduction in *Ccna2* level, similar to described in cardiomyocytes, can prevent cell division and result in inhibition of myogenesis (Fajas et al. 2001). In our experiment, TGF- β 1 caused decrease of *Ccna2* gene expression by 1.99-fold. (Table 1, position 32).

Bax (Bcl2-associated X protein) is a proapoptotic protein that belongs to the Bcl-2 family. In our experiment, TGF- β 1 caused a decrease of *Bax* gene expression by 1.87 fold (Table 1, position 46), which could lead to inhibition of cell apoptosis. This effect is analogous to described in the mouse fibroblasts (Zhang and Phan 1999). In this study TGF- β 1 had an antiapoptotic impact on these cells.

Mdfl (MyoD family inhibitor) is an inhibitor of MyoD proteins. It is assumed that inhibition of MyoD binding to DNA is associated with the interactions between *Creb1* and *Mdfl* factor. Kraut et al. found (1998) that *Mdfl* is a negative regulator of bHLH proteins. In addition, *Mdfl* can inhibit Wnt pathway target transcriptional factors such as LEF-1 and TCF (Pan et al. 2005). In our experiment, TGF- β 1 caused decrease of *Mdfl* gene expression by 1.98-fold (Table 1, position 36).

Tef (thyrotrophic embryonic factor) is a protein belonging to a subfamily of PAR proteins (proline and acidic amino acid-rich), that are bzip (basic region / leucine zipper). Data from the literature indicate the involvement of the TEF in the regulation of Ca^{2+} -dependent gene expression with participation of calmodulin and *Creb* as well as in antiapoptotic activity (Krueger et al. 2000). In our experiment, TGF- β 1 caused decrease of *Tef* gene expression by 1.88-fold (Table 1, position 104).

Tubg1 (tubulin, gamma 1) is a universal protein responsible for the organization of microtubule centers during cell division with the strongest effect in prophase and metaphase. In our experiment, TGF- β 1 caused decrease of *Tubg1* gene expression by 1.75-fold, that could lead to abnormalities in the organization of microtubules centers which resulted in preventing cell division and inhibition of myogenesis.

Cxcl5 (chemokine C-X-C motif ligand 5) is an inflammatory chemokine belonging to the CXC family. Results of the studies performed with the use of

DNA microarrays indicate that the *Cxcl5* gene is strongly expressed in proliferating chondrocytes and damaged muscle cells (James et al. 2005). In our experiment, TGF- β 1 caused decrease of *Cxcl5* gene expression by 2.36-fold (Table 1, position 206).

Rho (rhodopsin) is a transmembrane protein involved in signaling of integrins and EQF-PI3K collagen signaling, acting as a receptor dependent on G proteins. It is correlated with G proteins, together with the *Creb* protein (Gloriam et al. 2007). In our experiments, TGF- β 1 caused a decrease of *Rho* gene expression by 1.88-fold (Table 1, position 232). *Rho* protein is also known to be involved in the Wnt pathway. It is involved in the non-canonical Wnt pathway, so the decrease of its expression can cause disruption and inhibition of cell differentiation.

Calca (calcitonin/calcitonin-related polypeptide, α) is a large propeptide known as *Calcl* and is a product of the *Calca* gene. Calcitonin with parathormone and calcytriol is responsible for the regulation of calcium-phosphate metabolism. In our experiments, TGF- β 1 caused increase of *Calca* gene expression by 2.41-fold (Table 1, position 63). Further experiments need to be performed, but we hypothesize that this influenced the reduction of the level of Ca^{2+} in muscle cells which resulted in the inhibition of cell development.

Lgals4 (lectin, galactose binding, soluble 4) S-type lectin involved in the binding of other proteins and carbohydrates. It is a 323-amino acids protein demonstrating affinity to carbohydrates containing beta-galactosidose. In a study on the role of *Lgals4* in the development of colorectal cancer, it was found that the expression of this protein in tumors decreases between 1.5 and 50 times (Rechreche et al. 1997). Our experiment showed, that TGF- β 1 caused a decrease of *Lgals4* gene expression by 1.61 fold.

In our experiment, pathway analysis of regulated genes using *Panther* software revealed that they belong to 59 different signaling pathways but the majority of identified genes were involved in two partially covering signaling pathways: Wnt and cadherin signaling pathways (Fig. 5). These pathways play a significant role in the myogenesis processes such as transcriptional regulation, cell adhesion and migration (Kuang et al. 2008).

In the mammalian genome, there are 19 genes encoding Wnt proteins, which have a major impact on proliferation and differentiation. These factors influence the regulation of genes involved in cell growth, entry into cell cycle, proliferation, apoptosis, angiogenesis, embryonic development and cancerogenesis by binding to E-box sites and other sequences which could activate transcription. These molecules initiate several signal transduction pathways like the canonical Wnt, Wnt/ Ca^{2+} and Wnt/Planar Cell Polarity pathways (Eisenberg 2007). In our study Wnt sig-

naling pathway was represented by 12 down-regulated genes such as: *Pcdhgb8* ($\downarrow 1.89$), *Wnt16* ($\downarrow 2.02$), *Cttna11* ($\downarrow 2.01$), *Pcdhgc4* ($\downarrow 1.95$), *Pcdh16* ($\downarrow 1.99$), *Fat3* ($\downarrow 1.98$), *Dchs2* ($\downarrow 1.89$), *Pcdhb20* ($\downarrow 2.18$), *Pcdh1* ($\downarrow 2.08$), *Pcdhb17* ($\downarrow 1.68$), *Smad5* ($\downarrow 1.63$), *Hoxc6* ($\downarrow 2.28$). Wnt factors *in vivo* modulate myogenesis, preventing impaired cell adhesion and increasing concurrent protein transcription. The Wnt signaling pathway is also involved in the regulation of Myf5, MyoD, kinase A and Creb level of transcription during myogenesis. Wnt factors also influence the expression of Pax3 and Pax7 together with Wnt6, which enables β -catenin stabilization during c-myc activation (Buckingham 2006).

Cadherins are glycoproteins involved in cell adhesion associated with Ca^{2+} ions. These proteins have extracellular regions that mediate cell-cell interactions and play an important role in cell death, signaling, differentiation and migration. These proteins also have a transmembrane domain and a cytoplasmic tail that binds catenins. Catenins link the cadherin to the actin cytoskeleton and play a significant role in cellular signaling. α -Catenins bind indirectly to cadherins via interaction with β -catenin. In our study cadherin signaling pathway was represented by 10 down-regulated genes such as: *Pcdhgb8* ($\downarrow 1.89$), *Wnt16* ($\downarrow 2.02$), *ErbB4* ($\downarrow 2.06$), *Pcdhgc4* ($\downarrow 1.95$), *Pcdh16* ($\downarrow 1.99$), *Fat3* ($\downarrow 1.98$), *Dchs2* ($\downarrow 1.89$), *Pcdhb20* ($\downarrow 2.18$), *Pcdh1* ($\downarrow 2.08$), *Pcdhb17* ($\downarrow 1.68$).

The main path of signal transduction through the Wnt pathway is the canonical Wnt. To date, major signaling pathways downstream of the Frizzled (Fz) receptor have been identified including a canonical or Wnt/ β -catenin dependent pathway and the non-canonical or β -catenin-independent pathway which can be further divided into the Planar Cell Polarity and the Wnt/ Ca^{2+} pathways and these branches are now being actively investigated at the molecular and biochemical levels (Komiya 2008). The canonical pathway is activated when Wnt proteins bind to their cell membrane receptors, which are the 10 known members of the frizzled family of transmembrane proteins. Signal transduction from this ligand/receptor interaction occurs through the cytoplasmic protein Dishevelled (Dsh) which promotes the inactivation of glycogen synthase kinase 3 (GSK3). Absence of Wnt factors leads to degradation of cytoplasmic β -catenin and results in inhibition of gene transcription. The inhibition of GSK3 activity by the Wnt signal results in the cellular accumulation of β -catenin, which then translocates to the nucleus and forms a transcriptional enhancer complex with LEF/TCF DNA-binding proteins. Degradation of β -catenin occurs with contribution of 26S proteasome, serine-treonine kinases, CKI, GSK3, axin and APC protein (Polakis 2002). Our study indicates a significant influence of TGF- β 1 on genes involved in these proteins expression, with the

exception of APC and GSK3. Our results also indicate a strong decrease of α -catenin gene expression. α -Catenin binds to cytoplasmatic domain of cadherin type I and is responsible for organization and function of cadherins by binding them to actin of the cytoskeleton (Nelson et al. 2004). Integrity of structural cadherin-catenin complex is regulated by serine-treonine phosphorylation of β -catenin or epithelial cadherin (E-cadherin). Our study also showed a down-regulation of genes encoding α -catenin precursor proteins and ErbB4. ErbB4 protein is an important stimulator of cardiac muscle myogenesis and plays significant role in EGFR pathway. These results confirm the influence of TGF- β 1 on myogenesis through Wnt and cadherin signaling pathways (Garcia-Rivello et al. 2005).

Other interesting Wnt pathways involved in embryogenesis process are non-canonical: the Planar Cell Polarity pathway (PCP pathway) and the Wnt/ Ca^{2+} pathway. Those pathways are often referred as the β -catenin-independent. PCP pathway further modulates canonical signaling for dorsal axis formation and PCP signaling for gastrulation cell movements. The Wnt/ Ca^{2+} pathway emerged with the finding that some Wnts and Fz receptors can stimulate intracellular Ca^{2+} release from ER and this pathway is dependent on G-proteins (Komiya 2008). It is interesting because our studies indicated that TGF- β 1 could decrease expression of Rho protein engaged into PCP pathway and the level of Ca^{2+} -related proteins engaged into Wnt/ Ca^{2+} pathway such as calmodulin.

Other studies investigating the role of TGF- β 1 suggest that this cytokine can act independently of Wnt signaling pathway. It was found that an increase of expression of zinc finger Slug/Snail proteins can inhibit E-cadherin transcription and activation of FGFR, ErbB1 i ErbB2 (Peinado et al. 2003). The effect of increased Slug/Snail expression resulting in a loss of cell adhesion and an increase of migration, just in case of β -catenin accumulation, what can be an evidence of some independence from Wnt signaling pathway. In the studies mentioned above it was shown that Wnt signaling pathway regulates E-cadherin expression and that *Slug* could be a target gene for β -catenin/TCF complex. This complex could play a repressor function on E-cadherin promoter. Decrease of E-cadherin expression could impair cell adhesion and reduces the cells affinity to Wnt factors, which is a result of changes in β -catenin protein levels. Activation of transcription of target genes by β -catenin is coordinated with TGF- β 1 signaling pathway and connected with Smad proteins. Based on studies of TGF- β 1 and myostatin action it can be concluded that inhibition of C2C12 cells differentiation is mediated by c-Ski protein, which binds R-Smad protein (Budasz-Świdarska et al. 2005). It was also seen in

our studies, since we observed significant decrease of Smad5 expression. Smad5 is a receptor-regulated Smad (R-Smad), which is a transcriptional modulator activated by BMP (bone morphogenetic proteins) type 1 receptor kinase (Miyazono et al. 2003). However, with the level of significance used in our study it was not possible to show interdependence between levels of c-Ski, TGF- β 1 and myostatin. It has also to be mentioned that Smad proteins, TCF/LEF and catenin- β are able to exert an effect on transcription only when they are involved in those processes altogether (Labbe et al. 2000, Nishita et al. 2000). Variety of TGF- β 1 receptor interactions could intersect through Wnt pathway, regulate availability of β -catenin, break catenin-cadherin bonds and block cadherin expression (Nelson and Nusse 2004).

Conclusions

In conclusion, TGF- β 1 impairs the processes of genes transcription leading to inhibition of myogenic cell differentiation. The conclusions are based on facts that: 1) the majority of regulated genes were down-regulated; 2) three of eleven so-called key genes are genes strictly related with regulation of transcription, 3) TGF- β 1-regulated signaling pathways were Wnt and cadherin signaling pathways, which are closely cross-related and involved in the process of genes transcription. Myogenesis disturbances can be a result of decreased expression of factors directly taking part in processes of gene transcription such as *Max*, *Creb1*, *Creb5*, *Tef* and inhibiting the expression of genes encoding proteins, that modulate pathways involved in cells differentiation, such as *Wnt16*, *Ctnn11*, *Ccna2*, *Pdgfc* and protocadherins.

All of 502 identified genes are presented in Table 1 available at the website <http://www.sggw.info/knf/microarray/tgfb1/index.html>

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