PPARα does *not* suppress muscle-associated gene expression in brown adipocytes

Tomas B. Waldén, Natasa Petrovic, Jan Nedergaard*

Address: The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, SE-106 91 Stockholm, Sweden.

∗Corresponding author

Abstract

Brown adipocytes and myocytes develop from a common adipomyocyte precursor. PPARα is a nuclear receptor important for lipid and glucose metabolism. It has been suggested that in brown adipose tissue, PPARα represses the expression of muscle-associated genes, in this way potentially acting to determinate cell fate in brown adipocytes. To further understand the possible role of PPARα in these processes, we measured muscle-associated gene expression in brown adipose tissue and brown adipocytes from PPARα-ablated mice, including structural genes (Mylpf, Tpm2, Myl3 and MyHC), regulatory genes (myogenin, Myf5 and MyoD) and a myomir ((miR-206), as well as genes that are part of the brown adipocyte signature (Tbx15, Meox2, Zic1 and Lhx8). However, we report here that in our hands the expression of these genes was not influenced by the presence or absence of PPARα, nor by the PPARα activator Wy-14,643. Thus, it would not seem that PPARα plays a significant role in the regulation of the bifurcation of the adipomyocyte precursor into a brown adipocyte or myocyte phenotype; particulary, it would not seem that PPARα represses muscle-associated genes.

Keywords: PPARα, brown adipose tissue, myogenin, MyoD, miR-206, Mylpf, TPM2, Myl3

Introduction

A common origin of brown adipose tissue and skeletal muscle has now become established[1-4]. Both tissues derive from the dermomyotome and initially express myogenic transcription factors and microRNAs (myomirs). The master regulator "PR-domain containing 16" (Prdm16) down-regulates the expression of myogenic factors Myf6, MyoD, myogenin and MyHC in C2C12 muscle cell lines, thereby blocking progression into the myogenic program and facilitating brown adipocyte differentiation [3]. Prdm16 forms a complex with and coactivates the transcriptional function of the nuclear hormone receptor "perixosome proliferator activated receptor-α" (PPARα) [3]. In this respect, a remarkable observation by Tong et al. gains renewed interest[5]. In the brown adipose tissue of PPARα-ablated mice, these authors observed the presence of proteins normally associated with muscle[6, 7], including tropomyosin-β (Tpm2), myosin regulatory light chain 2 (Mylpf) and myosin light chain 3 (Myl3). Also the corresponding mRNA levels were reported to be higher, about 7-fold higher as a mean. Treatment with an PPARα agonist (Wy 14,643) further halved the expression level in the wild-type mice but was without effect in the PPARα-ablated mice. Taken together, these data strongly suggested that PPARα expression and activity in brown adipocytes repress the myogenic signature.

Thus, a scenario could be envisaged where Prdm16 performed its cell-fate determining effect (partly) by co-activating PPARα. Since PPARα would be a mediator for this effect, the myogenic pathway would be promoted in its absence.

In order to open for further exploration of this interesting possibility, we investigated in the present study the gene expression profiles of brown adipose tissue and brown adipocytes from PPARα-ablated mice, examining not only the expression of muscle structural genes (as those examined by Tong et al.) but also muscle myogenic regulatory factors and brown-fat specific regulatory factors. However, a repressive effect of PPARα on muscle-associated genes could not be observed.

Materials and Methods

Animals and tissue sampling. The experiments were approved by the animal ethical committee for North Stockholm. Male and female PPARα-ablated mice on a 129/Sv genetic background [8], and wildtype (+/+) 129/Sv mice 6-9 weeks old, from our own breeding, were killed with CO_2, and interscapular brown adipose tissue was dissected out and immediately frozen in liquid nitrogen, and later subjected to gene expression analysis. The genotype of the mice was confirmed by RT-PCR (Suppl. Fig. 1).

Cell isolation and cell culture. Male and female 3-4 weeks old PPARα-ablated mice on a 129/Sv genetic background and wildtype (+/+) 129/Sv mice from own breeding were killed with CO_2. BAT was isolated from the interscapular, cervical and axillary depots as described previously [9, 10]. The cells were cultured in 6-well plates (10 cm^2/well; Corning); 1.8 ml of culture medium was added to each well before 0.2 ml cell suspension was added. The culture medium was DMEM with 10 % (vol/vol) newborn calf serum (Hyclone batch APE21200) , 2.4 nM insulin, 25 µg/ml sodium ascorbate, 10 mM HEPES, 4 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 1 µM rosiglitazone maleate (Alexis Biochemicals). Cultures were washed in DMEM on day-1 and the medium was changed on day-1, day-3 and day-5. The media was not changed on the day the cells were harvested (day-7). Some cells were stimulated with 10 µM Wy-14,643 on day-5, 48 h prior to harvest.

RNA isolation, Northern blot and Real-Time qPCR. Total RNA was extracted from frozen brown adipose tissue or cell cultures with (Ultraspec Biotecx, Houston, TX) according to the manufacturer's protocol, and RNA concentrations were measured on a Nanodrop nd-1000 spectrophotometer(Thermo-Scientific,Wilmington, DE).

Aox1 mRNA levels were measured with Northern blot as described previously[11]. Membranes were probed for Aox1 mRNA and 18S rRNA after being stripped in-between by repeated washing with boiling 0.2% (wt/vol) SDS. Aox mRNA levels were normalised to 18S rRNA levels.

To synthesize cDNA, 200 ng RNA from each sample were reverse-transcribed with a High Capacity cDNA kit (Applied Biosystems, Foster City, CA) in a total volume of 10 µl and diluted to

200 µl. To measure each gene of interest (GOI), 4 µl of each cDNA sample were loaded in duplicate in SybrGreen® Jumpstart™ Taq Ready-mix (Sigma-Aldrich), together with pre-validated, designed primers (Suppl. Table 1) obtained from the Universal Probe Library (Roche Applied Science), on a 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA). All mRNA levels were normalized to TFIIB mRNA according to the comparative threshold method (Ct-method) (ΔCt). The TFIIB mRNA levels are provided in the Supplementary Material section (Suppl. Fig. 2) and were even between the genotypes both in-vivo and in-vitro. The equation $2^{-\Delta Ct}$ was applied to convert the logarithmic ΔCt values to linear values.

For miR-206 microRNA measurements, total RNA was diluted to5 ng/µl. In the microRNA-cDNA synthesis, 2.5 µl of these dilutions was reverse-transcribed in 3.5 µl reaction mix and 1.5 µl of the miR-206-specific reverse-transcription primer (Suppl. Table 1) provided with the MicroRNA Assay kit (Applied Biosystems, Foster City, CA). 1.2 µl of miRNA-specific cDNA from this reaction was amplified in duplicate with the TaqMan Gene Expression mastermix and the probe/primer mix provided in the MicroRNA Assay kit (Applied Biosystems, Foster City, CA) in the TaqMan qPCR system as above. TFIIB mRNA was used as an endogenous control; log to lin-transformed microRNA/TFIIB mRNA expression ratios were calculated as above.

Results

Muscle-associated genes in BAT are not suppressed by PPARα

In a 2D-SDS PAGE screen, Tong et al.[5] found high levels of five proteins in brown adipose tissue from PPARα-ablated mice, in comparison to wildtype tissue where these proteins were not at all detected. These proteins were identified by MALDI-TOF MS to be muscle-associated structural proteins (i.e. directly associated with muscle contractile function), namely tropomyosin 1 α-chain, tropomyosin 2 β, myosin regulatory light chain 2, myosin light chain 3 and parvalbumin α. By Northern blot techniques, the mRNA level of these genes was also found to be higher in PPARα-ablated mice than in wild-type mice.

Due to the potential significance of these findings for the understanding of the developmental biology of brown

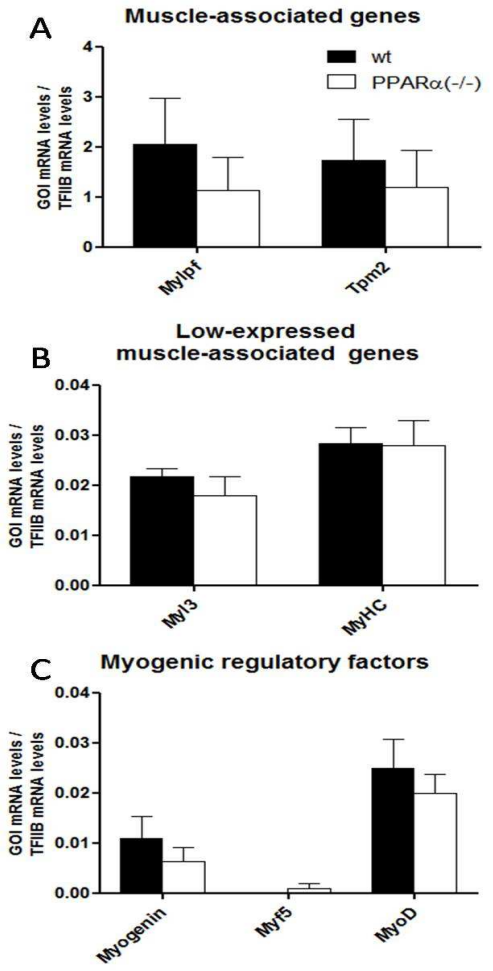

Fig. 1. Expression of muscle-associated genes and myogenic transcription factors in BAT. Interscapular brown adipose tissue mRNA levels of **(A)** relatively well-expressed muscle-associated structural genes (TPM2 and Mylpf), **(B)** low-expressed structural genes (Myl3 and MyHC), and **(C)** myogenic regulatory factors (myogenin, Myf5 and MyoD) were measured with Real-Time qPCR. Black and white bars represent data from wildtype and PPARα-ablated mice, respectively. The Ct-values for TPM2, Mylpf, Myl3, MyHC, myogenin, Myf5 and MyoD were normalized to TFIIB. The data are means ± SE from 7-8 mice. In soleus muscle tissue, Mylpf, Myf5 and myogenin mRNA levels were ~30-fold, ~40-fold and ~100-fold higher than the corresponding average levels in wild-type brown adipose tissue. In the wild-type group, data from one outlier mouse with a very high expression level was excluded.

adipocytes versus muscle cells, we have investigated the mRNA levels of three of these five proteins: tropomyosin 2 β (TPM2), myosin regulatory light chain 2 (Mylpf) and myosin light chain 3 (Myl3)

in brown adipose tissue from 6-9 week-old adult wildtype and PPARα-ablated mice.

Mylpf and Tpm2 genes were relatively well expressed in brown adipose tissue from wildtype mice (Fig. 1A), although the level of Mylpf was still about 30 times lower than in muscle (not shown). However, unexpectedly, the expression level of these genes in the brown adipose tissue of PPARα-ablated mice was not different from that in wild-type tissue. The expression level of Myl3 was as such much lower, but again, the mRNA levels were identical in brown adipose tissue from wildtype and PPARα-ablated mice (Fig. 1B).

In addition to these genes identified by Tong et al, we also measured myosin heavy chain (MyHC) mRNA levels. MyHC is one of the muscle-associated gene that was shown to be suppressed by Prdm16[3]. However, also MyHC mRNA levels in brown adipose tissue from wild-type and PPARα-ablated mice were equal (Fig. 1B).

Thus, the observation that the levels of the muscle-associated structural genes TPM2, Mylpf, Myl3 and MyHC were not altered by the presence or absence of PPARα does not support the contention that PPARα suppresses muscle-associated genes and in this way is directly involved in the brown adipocyte/myocyte bifurcation.

PPARα is not a suppressor of myogenic transcription factors

Tong et al. suggested that the influence of PPARα on the structural muscle-associated genes could be indirect, i.e. based on an effect of PPARα on the expression of myogenic transcription factors. Myogenin, Myf5 and MyoD are classical transcriptionfactors for myogenesis[12] but are also present in brown pre-adipocytes[2]. Myf6, MyoD and myogenin are suppressed by Prdm16[3]. To assess whether PPARα is involved in the repression of these genes (and perhaps thus in mediating the effect of Prdm16), we measured these regulatory genes in the brown adipose tissue from the wildtype and PPARα-ablated mice (Fig. 1C).

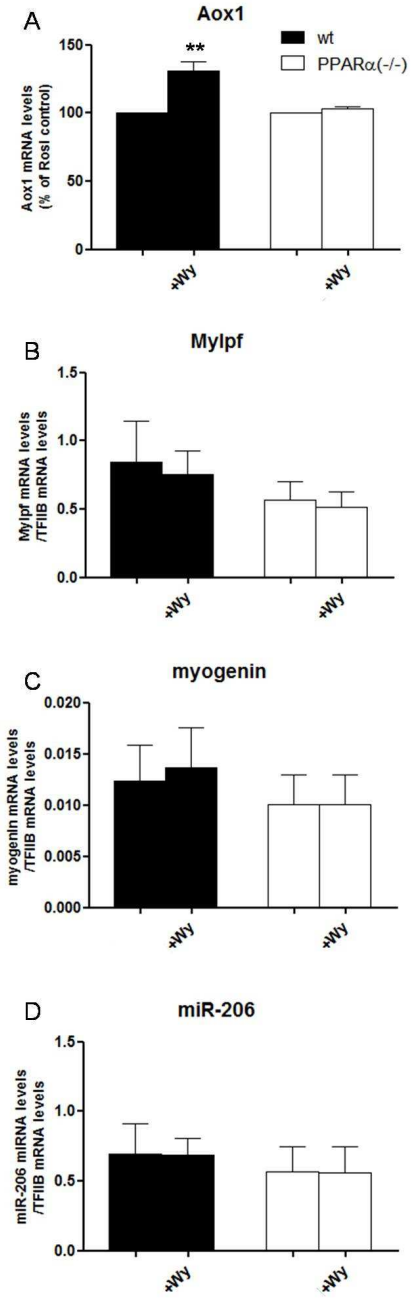

Fig. 2. Muscle-associated mRNA and microRNA levels in primary brown adipocytes. Levels of **(A)** Aox1 mRNA, **(B)** Mylpf mRNA, **(C)** myogenin mRNA and **(D)** miR-206 microRNA were measured in primary brown adipocytes with Northern blot (A) or Real-Time qPCR (B-D). Black bars indicate wildtype cells, white bars cells from PPARα-ablated mice; *Wy* indicates treatment with 10 μM of the PPARα experiment. In B-D, The Ct-values forMylpf, myogenin and miR-206 were normalized to TFIIB. Data shown are means ± SE from 5-6 independent cell culture experiments. **indicates a significant effect of Wy ($P < 0.01$).

Myf5 mRNA was almost absent, and myogenin and MyoD mRNA levels were low in brown adipose tissue (myogenin

about 100 fold lower than in muscle (not shown)). However, also these myogenic regulatory factors were equally expressed in brown adipose tissue from wildtype and PPARα-ablated mice.

Thus, PPARα has no regulatory effect on myogenic regulatory factor expression in brown adipose tissue.

Muscle-associated factors in PPARα null primary brown adipocytes

In a second model, we investigated the PPARα-mediated effect on muscle-associated mRNAs at a cellular level, utilizing primary brown adipocyte cultures from both wildtype and PPARα -ablated mice. Cultured brown adipocytes do not spontaneously express PPARα [10]. We therefore treated these cells with the PPARγ-ligand rosiglitazone to increase the PPARα levels[10, 13]. We further treated some of the cultures with a PPARα-agonist, Wy-14,643, to examine whether PPARα activation would augment the suggested effects of PPARα on myogenic gene expression, as was observed by Tong et al. in intact mice [5].

To verify that the Wy-14,643 treatment was efficient, we examined downstream PPARα -mediated effects.

Aldehyde oxidase 1 (Aox1) is a known target gene for PPARα (as well as for PPARγ[14]), and we used the expression of this gene to validate Wy-14,643-mediated PPARα activation (Fig. 2A). We could verify a slight Wy-14,643-response in the wildtype cells; the relatively small effect of Wy-14,643 is probably explainable in that Aox levels are already upregulated by rosiglitazone, mediated through PPARγ. In the brown adipocytes from the PPARα-ablated mice, there was no effect of Wy-14,643 on Aox1 gene expression. Thus, Wy-14,643 had an effect that was mediated via PPARα.

In line with the studies made in-vivo above, we measured Mylpf and myogenin mRNA levels in the brown adipocytes.

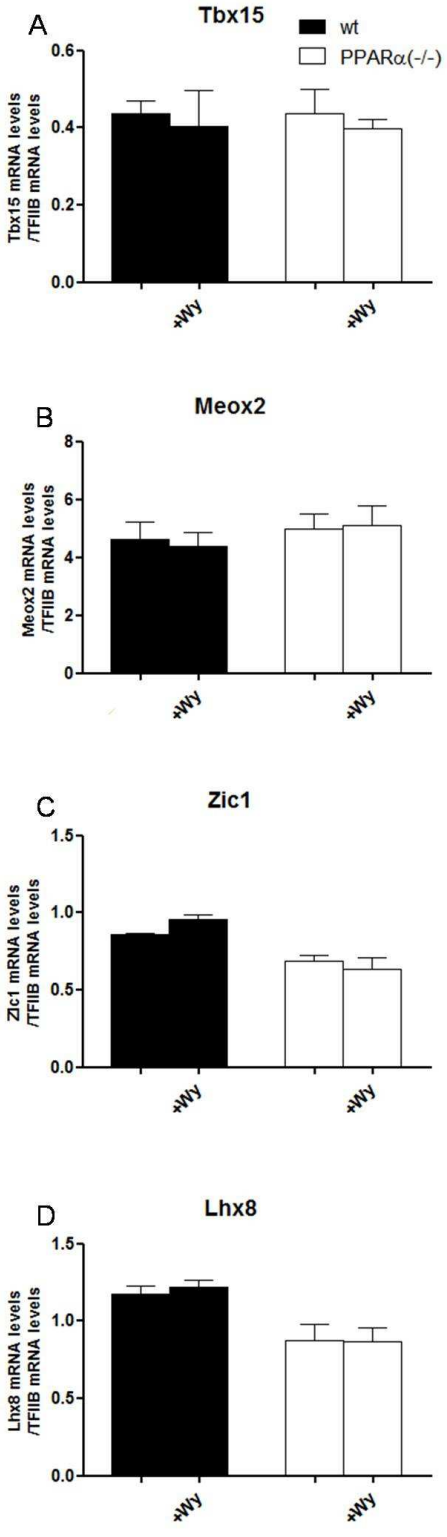

Fig. 3. Brown adipocyte marker mRNA levels in primary brown adipocytes. Levels of **(A)** Tbx15 mRNA, **(B)** Meox2 mRNA, **(C)** Zic1 mRNA and **(D)** Lhx8 were measured in primary brown adipocytes with Real-Time qPCR. Conditions and symbols as in Fig. 2.

Irrespective of the presence of a PPARα - activator or the presence or ablation of PPARα in these cultures, we found that the mRNA levels of these two muscle-associated genes were similar (Fig. 2BC).
In addition to muscle-specific mRNAs, muscle-specific microRNAs exist. These non-coding RNAs have been suggested to have pivotal roles in several cellular processes, in that they may silence networks of genes under the influence of of miR-206, a miRNA assumed to be specifically expressed in skeletal muscle[17]but recently demonstrated to be expressed also in brown adipocytes[4]. However, similarly to the Mylpf and myogenin results (Fig. 2BC), miR-206 levels were unaffected by PPARα loss or activation (Fig. 2D). This may be said to be in accordance with the other observations here, since Myf5 was almost not present and was not changed in the cells (Fig. 1C), and Myf5 is crucial for the induction of miR-206 expression[18].

Does PPARα influence brown adipocyte markers?
According to the hypothesis that PPARα regulates the expression of muscle-specific genes[5], the loss of PPARα would be expected to push the adipomyocyte-precursor towards a muscle phenotype, and thus to become less brown-fat-like. Therefore we examined the mRNA levels of four established brown adipocyte gene markers.

The transcription factors “T-box 15” (Tbx15) and “mesenchyme homeobox 2” (Meox2) are two genes found in both brown adipocytes and skeletal myocytes but not in white adipocytes[2].Tbx15 and Meox2 mRNA levels were not affected by either Wy-14,643 treatment or PPARα absence (Fig. 3AB).

In contrast, “zinc fingers in the cerebellum 1” (Zic1) and “Lim-homebox 8” (Lhx8) mRNAs are specifically found in brown adipocytes as compared to both muscle and white adipocytes[2], and if PPARα inhibits differentiaton towards muscle (and thus promotes differentiation towards brown adipocytes), these genes would be expected to be suppressed in the PPARα KO. However, these genes were also unaffected in all cases. The stable levels of these established brown adipocyte gene markers provide further support that PPARα does not control the bifurcation of the adipomyocyte-precursor towards myocytes versus brown adipocytes and does not have an impact on brown adipocyte identity.

Discussion

The mechanism that controls the bifurcation of the adipomyocyte-precursor into brown adipocytes or myocytes involves switching off or on muscle-specific factors, respectively. A role for PPARα in the switching mechanism was suggested by Tong et al.[5], even before it was understood that brown adipocytes and myocytes originate from a common adipomyocyte precursor. This suggestion gained renewed actuality when the relationship between myocytes and brown adipocytes was understood [1, 2] and especially when it was demonstrated that Prdm16 - that directs the adipomyocyte precursors towards the brown adipocytes phenotype - interacts directly with PPARα[3]. PPARα-ablated mice are essential tools to establish the functional roles of PPARα. In order to be able to extend the analysis of the significance of PPARα, we examined - similarly to Tong et al. - muscle-associated gene expression in the brown adipose tissue of PPARα-ablated mice and in brown adipocyte cultures from these mice.

However, in contrast to the observations and suggestions of Tong et al. concerning the significance of PPARα for the expression of muscle-associated genes, we found *no* increased expression of muscle-associated gene regulatory factors. Thus, although demonstrated to interact with PRDM16[3], and despite the observations of Tong et al., PPARα does not seem to be involved in defining the brown adipocyte by suppression of myogenesis. These observations concerning the mRNA levels of muscle-associated genes in the brown adipose tissue of PPARα-ablated mice are clearly not concurrent with the observations of Tong et al.[5]. We are unable to suggest a simple explanation for this major difference. The PPARα-ablated mice studied by us come from the same founders as those studied by Tong et al., and both direct examination of the genome (Suppl. Fig. 1) and functional analysis of PPARα agonist effects (Fig. 2) confirm

that PPARα is ablated even in our mouse colony. A source of error could be contamination during dissection by surrounding muscular tissues in the studies of Tong et al. However, Tong et al. state that based on 2D SDS-PAGE analysis, the excised brown adipose tissue was not contaminated by other tissues (although the experimental background for this statement is not detailed). It is also difficult to understand why a contamination would be systematically biased to the PPARα mice.

In conclusion, it would not seem that a PPARα inhibitory effect on the myogenic program, that would force the adipomyocyte towards a brown adipocyte, is relevant for an understanding of the adipomyocyte bifurcation.

Aknowledgements

The authors thank Sofie Wagenius for animal breeding and Jeanette Johansen at the Center for Molecular Medicine, Karolinska Institute for access to the 7900HT Real-Time qPCR instrument. This study was supported by grants from the Swedish Research Council. The authors participate in the EU-programmes Mitofood and ADAPT.

References

[1] R. Atit, S.K. Sgaier, O.A. Mohamed, M.M. Taketo, D. Dufort, A.L. Joyner, L. Niswander, R.A. Conlon. Beta-catenin activation is necessary and sufficient to specify the dorsal dermal fate in the mouse, Dev. Biol. 296 (2006) 164-176.

[2] J.A. Timmons, K. Wennmalm, O. Larsson, T.B. Walden, T. Lassmann, N. Petrovic, D.L. Hamilton, R.E. Gimeno, C. Wahlestedt, K. Baar, J. Nedergaard, B. Cannon. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4401-4406.

[3] P. Seale, B. Bjork, W. Yang, S. Kajimura, S. Chin, S. Kuang, A. Scime, S. Devarakonda, H.M. Conroe, H. Erdjument-Bromage, P. Tempst, M.A. Rudnicki, D.R. Beier, B.M. Spiegelman. PRDM16 controls a brown fat/skeletal muscle switch, Nature 454 (2008) 961-967.

[4] T.B. Walden, J.A. Timmons, P. Keller, J. Nedergaard, B. Cannon. Distinct expression of muscle-specific microRNAs (myomirs) in brown adipocytes, J. Cell. Physiol. 218 (2009) 444-449.

[5] Y. Tong, A. Hara, M. Komatsu, N. Tanaka, Y. Kamijo, F.J. Gonzalez, T. Aoyama. Suppression of expression of muscle-associated proteins by PPARalpha in brown adipose tissue, Biochem. Biophys. Res. Commun. 336 (2005) 76-83.

[6] Y. Wang, D. Szczesna-Cordary, R. Craig, Z. Diaz-Perez, G. Guzman, T. Miller, J.D. Potter. Fast skeletal muscle regulatory light chain is required for fast and slow skeletal muscle development, FASEB J. 21 (2007) 2205-2214.

[7] J.C. Sanchez, D. Chiappe, V. Converset, C. Hoogland, P.A. Binz, S. Paesano, R.D. Appel, S. Wang, M. Sennitt, A. Nolan, M.A. Cawthorne, D.F. Hochstrasser. The mouse SWISS-2D PAGE database: A tool for proteomics study of diabetes and obesity, Proteomics 1 (2001) 136-163.

[8] S.S. Lee, T. Pineau, J. Drago, E.J. Lee, J.W. Owens, D.L. Kroetz, P.M. Fernandez-Salguero, H. Westphal, F.J. Gonzalez. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators, Mol. Cell. Biol. 15 (1995) 3012-3022.

[9] M. Nechad, P. Kuusela, C. Carneheim, P. Bjorntorp, J. Nedergaard, B. Cannon. Development of brown fat cells in monolayer culture. I. morphological and biochemical distinction from white fat cells in culture, Exp. Cell Res. 149 (1983) 105-118.

[10] N. Petrovic, I.G. Shabalina, J.A. Timmons, B. Cannon, J. Nedergaard. Thermogenically competent non-adrenergic recruitment in brown predipocytes by a PPAR{gamma} agonist, Am. J. Physiol. Endocrinol. Metab. (2008) .

[11] E.M. Lindgren, R. Nielsen, N. Petrovic, A. Jacobsson, S. Mandrup, B. Cannon, J. Nedergaard. Noradrenaline represses PPAR (peroxisome-proliferator-activated receptor)

gamma2 gene expression in brown adipocytes: Intracellular signalling and effects on PPARgamma2 and PPARgamma1 protein levels, Biochem. J. 382 (2004) 597-606.

[12] M. Buckingham. Muscle differentiation. which myogenic factors make muscle? Curr. Biol. 4 (1994) 61-63.

[13] N. Petrovic, T.B. Walden, I.G. Shabalina, J.A. Timmons, B. Cannon, J. Nedergaard. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes, J. Biol. Chem. 285 (2010) 7153-7164.

[14] T. Shiraki, N. Kamiya, S. Shiki, T.S. Kodama, A. Kakizuka, H. Jingami. Alpha,beta-unsaturated ketone is a core moiety of natural ligands for covalent binding to peroxisome proliferator-activated receptor gamma, J. Biol. Chem. 280 (2005) 14145-14153.

[15] Y.M. Shah, K. Morimura, Q. Yang, T. Tanabe, M. Takagi, F.J. Gonzalez. Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation, Mol. Cell. Biol. 27 (2007) 4238-4247.

[16] T. Sun, M. Fu, A.L. Bookout, S.A. Kliewer, D.J. Mangelsdorf. MicroRNA let-7 regulates 3T3-L1 adipogenesis, Mol. Endocrinol. 23 (2009) 925-931.

[17] C. Anderson, H. Catoe, R. Werner. MIR-206 regulates connexin43 expression during skeletal muscle development, Nucleic Acids Res. 34 (2006) 5863-5871.

[18] D. Sweetman, K. Goljanek, T. Rathjen, S. Oustanina, T. Braun, T. Dalmay, A. Munsterberg. Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133, Dev. Biol. 321 (2008) 491-499.