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Concomitant modulation of transcripts related to fiber type determination and energy metabolism in skeletal muscle of female ovariectomized mice by estradiol injection☆

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ABSTRACT

In postmenopausal women, prevalence of metabolic syndrome (MS) is 40%. Aging is associated with a decline in basal metabolic rate and an alteration in tissue metabolism, leading to MS. Hormonal therapy has been shown to be effective against some of the MS-related features but its effects on sarcopenia and skeletal muscle metabolism remain unclear. We have analyzed the effects of estradiol (E_2) on global gene expression in skeletal muscle of ovariectomized (OVX) female C57BL6 mice using the serial analysis of gene expression method. Animals were randomly assigned to six groups of each 14 mice: the vehicle group (OVX), and five groups in which E_2 was injected 1 h, 3 h, 6 h, 18 h or 24 h prior to sacrifice. E_2 modulated 177 transcripts, including 11 partially characterized transcripts and 52 potentially novel transcripts. Most of the differentially expressed transcripts were up-regulated at E_2 3 h and E_2 18 h, while down-regulated transcripts were observed at E_2 6 h and E_2 24 h, illustrating two cycles of up and down E_2-responsive genes. Modulated transcripts were involved in skeletal muscle structure/growth, fiber type distribution and energy metabolism. These results suggest that a single physiological dose of E_2 can concomitantly modulate transcripts determining skeletal muscle type and energy metabolism, which may in turn affect sarcopenia and MS.

1. Introduction

Aging is a natural process that every human being must go through. In women, hormonal changes and menopause are hallmarks of the aging phenomenon. Since life expectancy is increasing, the time a woman spends after menopause can constitute one-third of her life. Menopause is defined as the permanent cessation of menses, which results in a state of estrogen deficiency. In the USA, more than 20% adults suffer from metabolic syndrome (MS), whereas in elderly and postmenopausal women, the prevalence of MS has reached 40% [1]. Moreover, the postmenopausal status is associated with 60% increased risk of MS [1]. MS is a constellation of risk factors increasing the risk of developing cardiovascular heart disease (CVD) and diabetes. In women, estrogen depletion is partially responsible for the emergence of some of the MS-related features, such as abdominal adiposity, insulin resistance and dyslipidemia [2]. With the aging process, women tend to lose lean body mass, gain fat mass, and develop more abdominal obesity, the latter being associated with increased insulin resistance and atherosclerotic vascular disease.

Hormonal therapy (HT) taken following menopause has been shown to be effective to reduce waist circumference, visceral adipose tissue (VAT) and insulin resistance, as well as to improve lipoprotein profile, stabilize bone mass and prevent osteoporotic fractures [3]. However, several studies show that HT has important undesirable secondary effects. The well-known study conducted by the Writing Group for the Women's Health Initiative (WHI) Investigators [4] stated that overall health risks exceeded benefits from use of combined HT for healthy postmenopausal women. Indeed, HT increases risks of stroke, venous thromboembolism, breast cancer and endometrial cancer. Therefore, the authors concluded that it should not be given as preventive therapy for treating MS and preventing CVD.

Human skeletal muscle tissue comprises approximately 40–45% of total body weight, and is one of the most metabolically active tissues in the body. Thus, skeletal muscle accounts for more than 30% of basal metabolic rate (BMR). In women, fat-free mass remains relatively stable during premenopausal years whereas loss of muscle mass, sarcopenia, increases rapidly after menopausal transition [5]. Consequently, postmenopausal women display decreased 24 h

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energy expenditure and an increased VAT [6]. In addition to the sarcopenia, aging affects muscle fiber type composition, which in turn influences fuel utilization in the tissue and, consequently, insulin sensitivity [7]. The classification of muscle fiber type is based on the myosin heavy chain (MHC) isoform. In human adult skeletal muscle, three MHC isoforms can be found: MHC-I (slow or type I muscle fiber), MHC-IIa (fast oxidative or type IIa) and MHC-IIx/IId (fast glycolytic or type IIx/IId), in which the latter being the fastest myosin [8]. Type IIx muscle fiber was previously called type IIb in human [8], although it coexists with IIx in other mammals such as rodents. With aging, there is a reduction of number and size of type II muscle fibers as opposed to type I muscle fibers [9], which results in a relatively higher percentage of type I muscle fibers [7,10]. It is also known that obese individuals and diabetic patients show relatively lower type I muscle fiber [11,12], and individuals with relatively higher type II muscle fibers show lower body fat oxidation and higher insulin resistance [12]. Therefore, the changes in muscle fiber composition induced by aging appear to favor an increase in oxidative capacity. However, aging reduces muscle mitochondrial oxidative capacity and ATP production [7]. Moreover, skeletal muscle from elderly individuals shows a common phenotype, an impaired skeletal muscle oxidation, as seen from Duchenne muscular dystrophy [13], probably in part due to the sarcopenia. To our knowledge, no study has been conducted to investigate the early effects of HT on muscle fiber type composition in human. In ovariectomized (OVX) rats as a model of estrogen deficiency, 17β-estradiol (E_2) replacement for 4 weeks decreases type IIb muscle fibers in the plantaris (composed at 92% of fiber type II) [14] whereas no effect is seen in the soleus (composed at 85% of fiber type I) [15], thus suggesting that the response to E_2 appears to vary depending on the initial fiber type composition of the muscle [14]. Up to date, the effects of HT on sarcopenia still remain with controversy, and the molecular mechanisms of E_2-induced changes in skeletal muscle metabolism as well as its relation to muscle fiber composition are poorly understood. We have focused on gene regulation occurring within 24 h since genes responding to E_2 within this time period would be enriched in direct estrogen targets before cell growth.

With the advent of the serial analysis of gene expression (SAGE) strategy [16], we have been investigating the transcriptomic changes induced by sex steroids in skeletal muscle and VAT in order to identify direct target genes of the steroid hormones [17–20]. These studies have illustrated the molecular mechanisms responsible for the steroid actions in those tissues. Therefore, in order to characterize the molecular actions of E_2 in the skeletal muscle, the current study investigates the early transcriptomic changes in the gastrocnemius muscle of OVX mice following a single injection of a physiological dose of E_2.

2. Materials and methods

2.1. Sample preparation

The right gastrocnemius muscle was obtained from 84 C57BL6 mice, 12–14-week old, purchased from Charles River Canada Inc. (St-Constant, QC). The gastrocnemius muscle was selected among the different muscles since it is representative and composed of different fiber types [21,22]. The animals were housed with light on from 07:15 h to 19:15 h and had access to Lab Rodent Diet No. 5002 (Ren's Feed and Suppliers, ON) and water ad libitum. OVX was performed 7 days prior to sacrifice in the OVX and the 5 OVX + E_2 groups. Prior to sacrifice, mice of the OVX group ($n = 14$) received an i.p. injection of vehicle solution (0.4% (w/v) Methocel A15LV Premium/5% ethanol) 24 h before sacrifice, while a physiological dose of E_2 (50 ng/mouse) was injected 1, 3, 6, 18 and 24 h to the mice of E_2 1 h, E_2 3 h, E_2 6 h, E_2 18 h and E_2 24 h groups ($n = 14$ in each group), respectively. The mice of all the groups were alternatively sacrificed between 08:30 and 12:30 in the morning, by cervical dislocation and decapitation under isoflurane anesthesia. The right gastrocnemius muscle was sampled from each mouse and pooled together for analysis of the same group to eliminate inter-individual variations and to extract sufficient amount of mRNA. The tissues were stored at −80 °C until RNA extraction. All animal experimentation was conducted in accord with the requirements of the Canadian Council on Animal Care.

2.2. Transcriptome analysis

The SAGE method was performed as previously described [16,23]. Total RNA was isolated by Trizol (Invitrogen Canada Inc., Burlington, ON). The quality of total RNA was monitored by microcapillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, ON). Polyadenylated RNA was extracted with the Oligotex mRNA Mini Kit (Qiagen Inc., Mississauga, ON), annealed with the biotin-5′-T_{18}-3′ primer and converted to cDNA using the cDNA synthesis kit (Invitrogen Canada Inc.). The resulting cDNAs were digested with NlaIII (New England BioLabs Ltd., Pickering, ON) and the 3′ restriction fragments were isolated with streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, WI) and separated into two populations. Each population was ligated to one of two annealed linkers and extensively washed to remove unligated linkers. The tag beside the most 3′ NlaIII restriction site (CATG) of each transcript was released by digestion with BsmFI (New England BioLabs Ltd.). The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR and digested with NlaIII. The band containing the ditags was extracted from the 12% polyacrylamide gel with Spin-X microcentrifuge tube (Fisher, Pittsburgh, PA). The purified ditags were self-ligated to form concatemers using T4 ligase (Invitrogen Canada Inc.). The concatemers ranging from 500 bp to 1800 bp were isolated by agarose gel and extracted with Gene-Clean Spin (Qbiogene, Montreal, QC). The resulting DNA fragments were ligated into the SphI site of pUC19 and cloned into UltraMAX DH5αFT competent cell (Invitrogen Canada Inc.). White colonies were picked up with a Q-Bot colony picker (Genetix Ltd., Hampshire, UK). Concatemer inserts were sequenced by the Applied Biosystems 3730 (Foster City, CA). In general, variations of gene expression are followed by parallel protein amount and protein-associated function variations [24]. However, this is not always the case, since several mechanisms, such as post-transcriptional modifications, may occur. Nevertheless, the level of gene expression correlates well with the quantity of protein and their activity ($r = 0.94$, between SAGE and proteomics results) [25].

2.3. Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [26]. In brief, tags corresponding to linker sequences were discarded and replicate concatemers were counted only once. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11 bp tags) with SAGEmap, UniGene and GenBank databases. A minimum of one expressed sequence tags (EST) with a known polyA tail had to be in the UniGene cluster to identify the last NlaIII site on the corresponding cDNA. We have previously shown that the SAGE method is very reproducible with $r^2 = 0.96$ between two libraries constructed from the same total RNA pool [26]. Classification of the transcripts was based upon the updated information of the genome directory [27] as well as previously published literatures.

2.4. Validation by quantitative real-time PCR (Q_RT-PCR)

First-strand cDNA was synthesized using 5 μg of pooled RNA of each experimental group in a reaction containing 200 U of Superscript III RNase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 mM deoxynucleotides triphosphate, 5 mM dithiothreitol and 34 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, NJ) in a final volume of 50 μl. The resulting products after treating with RNase A were purified with Qiaquick PCR purification kits (Qiagen Inc.). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, NJ). Reagents were obtained from the same company and were used as described by the manufacturer. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, AB) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: apolipoprotein B editing complex 2 (Apobec2), NM_009694, 648–889; cullin 4a (Cul4a), NM_146207, 2309–2515; fatty acid binding protein 3 muscle and heart (Fabp3), NM_010174, 206–422; muscle glycogen phosphorylase (Pygm), NM_011224, 2517–2633; myozenin 1 (Myoz1), NM_021508, 231–460; myosin heavy polypeptide 2 (Myh2), NM_001039545, 5779–5911; malate dehydrogenase 2 NAD (mitochondrial) (Mdh2), NM_008617, 844–1007. The expression levels of mRNA (number of copies/μg total RNA) were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity. The standard curve was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) and a LightCycler 3.5 program provided by the manufacturer (Roche Inc.). The Q_RT-PCR was performed in triplicates. Data were expressed as a ratio to the Hprt1.

2.5. Statistical analysis

We used the comparative count display (CCD) test to identify the transcripts significantly differentially expressed ($p \leq 0.05$) between the groups with more than a 2-fold change [28]. The data were normalized to 100,000 SAGE tags for presentation.

We also performed χ^2 test to identify the significant differences in distributions on total number of SAGE tags corresponding to each myosin heavy polypeptide (Myh) isoform ($p < 0.05$). The data of Q_RT-PCR were analyzed by Student's t-test ($p < 0.05$).

3. Results

By sequencing approximately 130,000 SAGE tags in each experimental condition, we identified a total of 177 E_2-responsive genes/transcripts (ERGs), including 11 partially characterized and 52 potentially novel ERGs (Supplemental Table 1). In the characterized ERGs, $E_2$1 h, $E_2$3 h, $E_2$6 h, $E_2$18 h and $E_2$24 h had 7, 15, 35, 15 and 81 ERGs, respectively. Up-regulated ERGs were mostly observed in $E_2$3 h (Fig. 1A) and $E_2$18 h, whereas down-regulated ERGs were mostly seen in $E_2$6 h and $E_2$24 h (Fig. 1B). In which, 53% of $E_2$18 h-ERGs and 66% of $E_2$6 h-ERGs showed similar expression pattern as $E_2$3 h and $E_2$24 h, respectively, illustrating two cycles of up- and down-ERGs waves.

In $E_2$1 h, three ERGs related to magnesium transport, oxidative phosphorylation (OxPhos) and protein synthesis were suppressed, whereas one involved in cell proliferation was induced.

In $E_2$3 h, the up-regulated ERGs were mainly involved in contractile apparatus, OxPhos and ATP synthesis, calcium (Ca^{2+}) and ion transport, cell signaling, cell proliferation and apoptosis. A homeostasis-related ERG, carbonic anhydrase 3 (Car3), as well as Ca^{2+}-related transcripts were also induced at this time point. These metabolic changes were reflected by an increase in a total SAGE tag number corresponding to oxidative fibers (type IIa), although a significant increase was also noted for fiber type IIx (Fig. 2). On the other hand, one characterized ERG was down-regulated, glycosylation dependent cell adhesion molecule 1 (Glycam1) which is involved in cell adhesion.

In $E_2$6 h, the majority of repressed ERGs were related to energy metabolism and protein synthesis. Other ERGs involved in contractile apparatus, immunity, homeostasis, cell proliferation and cell differentiation were also down-regulated. A few induced ERGs in $E_2$6 h were related to the pentose-phosphate pathway in the sugar metabolism, to protein synthesis and to extracellular matrix. A significant decrease in the SAGE tag number for fast type fiber (IIx) also occurred (Fig. 2).

In $E_2$18 h, most of ERGs were induced. This is especially the case for cell signaling ERG protein kinase C substrate 80K-H (Prkcsh), which regulates positively glucose transport [29]. Other up-regulated ERGs were involved in OxPhos, Ca^{2+} transport, contractile apparatus, cells signaling, transcription, homeostasis, and immunity. As for the SAGE tag number (Fig. 2), there was a significant increase for fiber type IIx. Two ERGs, namely EST solute carrier family 25 and 16S ribosomal RNA were down-regulated.

$E_2$24 h was the time point in which the highest number of ERGs was observed. The repressed ERGs were related to fast type skeletal muscle as well as energy metabolism (sugar, lipid and amino acid metabolisms, OxPhos, and mitochondrial ATP synthesis). These metabolic changes were reflected by a decrease in a total SAGE tag number corresponding to type IIx (Fig. 2). Other down-regulated ERGs were involved in protein synthesis, cell signaling, cell differentiation, cell proliferation and growth, transcription, homeostasis and Ca^{2+} transport. On the other hand, only 11% of ERGs in $E_2$24 h were induced, including those related to pentose-phosphate pathway, protein synthesis and cell differentiation.

The results of Q_RT-PCR on Apobec2, Cul4a, Fabp3, Pygm, Myoz1, Myh2 and Mdh2 were concordant with the SAGE results (Fig. 3).

4. Discussion

Skeletal muscle is largely involved in energy metabolism and insulin sensitivity. HT has been shown to be effective against MS-related features such as abdominal adiposity, insulin resistance and blood pressure in postmenopausal women [3]. Insulin resistance is also a condition associated with decreased glucose uptake in insulin-sensitive tissues such as skeletal muscle [30].

4.1. Skeletal muscle structure and growth

Very few studies have investigated the effects of E_2 on MHC isoforms [14,15,31]. OVX alone appears to induce a shift in muscle fiber type from fast to slow type [31] and to cause a diminution in the relative amount of MHC-IIx [14]. Some studies have reported that long-term administration of E_2 in OVX animals had no influence on MHC isoforms [20,31], while Piccone et al. [14] have reported a reverse effect of E_2 on the diminished amount of MHC-IIx in OVX rats. In the present study, a single injection of E_2 induced transcripts corresponding to MHC-IIa and -IIx after 3 h as well as MHC-IIx after 18 h. On the other hand, E_2 repressed those for MHC-IIa after 6 h as well as MHC-IIa and IIx after 24 h. These results indicate that the effect of a single injection of E_2 on transcripts determining the muscle fiber type is not one direction, but rather two cycles of up- and down-waves. These observations do not appear to be the result of circadian rhythms since the mice of all the groups were

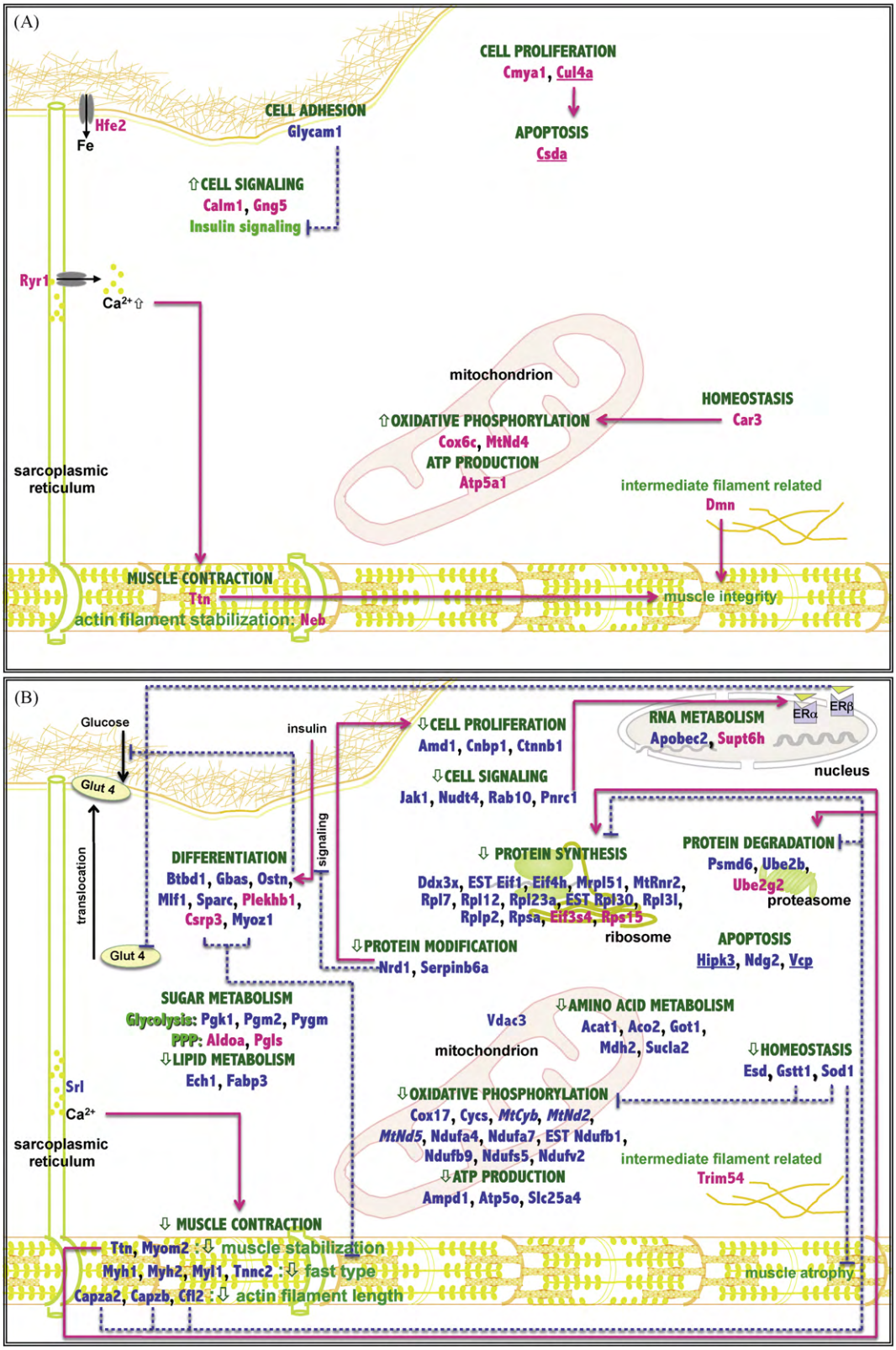

Fig. 1. Effects of estradiol injection at 3 h (A) and 24 h (B) on transcripts expressed in the skeletal muscle. The up- and down-regulation of the transcripts are shown in pink (solid arrow) and blue (dotted line), respectively. Underlined transcripts correspond to negative regulation/inhibition. *Abbreviations*: Acat1: acetyl-coenzyme A acetyltransferase 1; Aco2: aconitase 2; Aldoa: Aldolase 1, A isoform; Amd1: S-adenosylmethionine decarboxylase 1; Ampd1: adenosine monophosphate deaminase 1 (isoform M); Apobec2: apolipoprotein B editing complex 2; Atp5a1: ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1; Atp5o: ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit; Btbd1: BTB (POZ) domain containing 1; Ca^{2+}: calcium; Calm1: calmodulin 1; Capza2: EST capping protein (actin filament) muscle Z-line, alpha 2; Capzb: capping protein (actin filament) muscle Z-line, beta; Cfl2: EST cofilin 2, muscle; Car3: carbonic anhydrase 3; Cmya1: cardiomyopathy associated 5; Cnbp1: cellular nucleic acid binding protein 1; Cox6c: EST transcribed locus, strongly similar to NP_444301.1, cytochrome *c* oxidase, subunit VIc; Cox17: cytochrome *c* oxidase, subunit XVII assembly protein homolog (yeast); Csda: cold shock domain protein A; Csrp3: cysteine and glycine-rich protein 3; Ctnnb1: catenin beta; Cul4a: Cullin 4A; Cycs: cytochrome *c*, somatic; Ddx3x: fibroblast growth factor inducible 14; Dmn: desmuslin; Ech1: enoyl coenzyme A hydratase 1, peroxisomal; Eif1: similar to eukaryotic

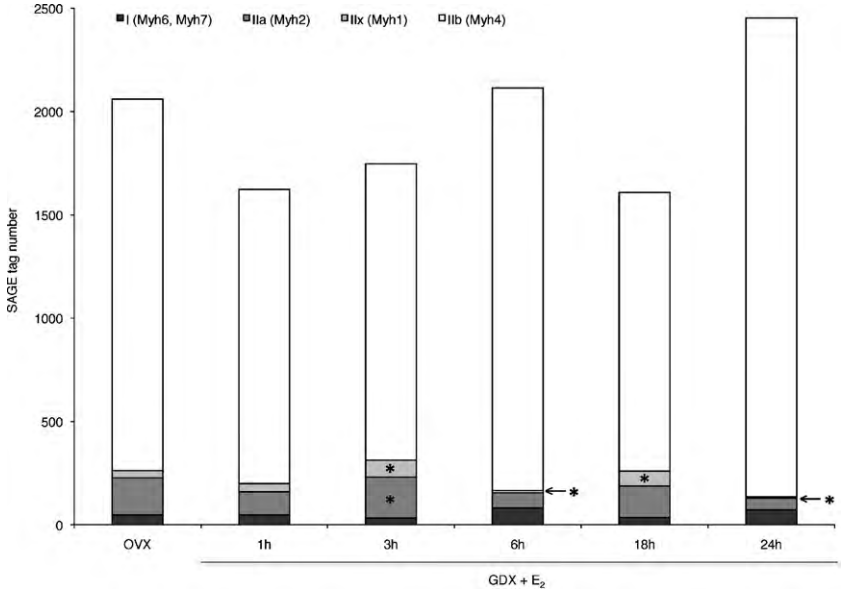

Fig. 2. Effects of estradiol on fiber type distribution. *Abbreviations*: E_2: 17β-estradiol; Myh: myosin heavy polypeptide; OVX: ovariectomized; SAGE: serial analysis of gene expression.

alternatively sacrificed between 08:30 and 12:30 in the morning. In addition, E_2 has an anorexigenic effect [32], and caloric restriction is associated with transcriptomic changes [33]. Thus, we cannot eliminate the possibility that E_2 modulated food intake, which in turn could modify gene expression in this study. However, the gene expression patterns at $E_2$24 h and $E_2$18 h clearly differ one from another. Mice were killed in the morning and are known to feed themselves at night. If the effect of food intake is larger than the one of E_2, their gene expression patterns should be similar. Therefore, the effect of food intake must be minimal. E_2 also possesses negative- and positive-feedback actions that cause changes at the level of the central nervous system [34]. In this study, it is not possible to distinguish central-mediated effects from direct effects on skeletal muscle. Thus, the reported two cycles of up- and down-waves may include the central effects. Accumulations of the length and wide of these waves within 24 h might contribute to the determination of muscle fiber type when E_2 was administrated for a several days or weeks. In the long-term E_2 administration, it has been proposed that E_2 could have a different effect on MHC isoforms in immature (growing) or mature skeletal muscle [14,15] and that the effect could change according to the initial fiber type composition of the muscle [15]. Other important aspects concern route and dose of administration and treatment length. In the present study, E_2 had no effect on total SAGE tag number of slow fibers. McCormick et al. [15] have also reported no effect of long-term E_2 administration on pronominally slow fiber type in soleus muscle [15]. Thus, the effects of E_2 on slow fiber type may be minimum.

4.2. E_2 effects at 24 h

Both MHC and myosin light chain isoforms determine the maximum velocity of contraction of skeletal muscle fibers [15]. In the present study, E_2 down-regulated four transcripts expressed in fast muscle fiber, namely Myh1 and Myh2, corresponding to MHC-IIx and MHC-IIa, respectively, as well as myosin light polypeptide 1 (Myl1) and troponin C2. In addition to these transcripts, E_2 repressed three transcripts, namely capping proteins alpha and beta and cofilin 2, which regulate actin filament turnover [35,36], as well as titin (Ttn) and myomesin 2. Titin is responsible for establishing and maintaining the structure and elasticity of sarcomeres in striated muscle, thus keeping the thick filament centered in the sarcomere [37]. Myomesin is a structural component essential for sarcomere stability, interacts with titin, and attaches the myosin thick filament to the M-line [38,39]. Our results suggest that E_2 may decrease fast type fiber expression and assembly of sarcomeres at 24 h after E_2 injection. E_2 also down-regulated Myoz1, a protein that belongs to the calsarcins family and that is especially expressed in fast muscle fibers [40]. Calsarcins interacts with

translation initiation factor 1 (eIF1); EST: expressed sequence tag (EST); Eif3s4: eukaryotic translation initiation factor 3, subunit 4 (delta); Eif4 h: Williams–Beuren syndrome chromosome region 1 homolog (human); ER: estrogen receptor; Esd: esterase D/formylglutathione hydrolase; Fabp3: fatty acid binding protein 3, muscle and heart; Fe: iron; Gbas: glioblastoma amplified sequence; Glut 4: glucose transporter 4; Glycam1: glycosylation dependent cell adhesion molecule 1; Gng5: guanine nucleotide binding protein (G protein) gamma 5 subunit; Got1: glutamate oxaloacetate transaminase 1, soluble; Gstt1: EST glutathione S-transferase, theta 1; Hfe2: hemochromatosis type 2 (juvenile); Hipk3: EST homeodomain interacting protein kinase 3; Jak1: janus kinase 1; Mdh2: malate dehydrogenase 2, NAD; Mlf1: myeloid leukemia factor 1; Mrpl51: EST mitochondrial ribosomal protein L51; MtCyb: cytochrome *b*; MtNd: NADH dehydrogenase subunit; MtRnr2: 16S ribosomal RNA; Myh1: myosin, heavy polypeptide, skeletal muscle, adult; Myl1: myosin, light polypeptide 1; Myom2: myomesin 2; Myoz1: myozenin 1; Ndg2: Nur77 downstream gene 2; Ndufa4: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4; Ndufa7: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (B14.5a); Ndufb1: EST NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1; Ndufb9: NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9; Ndufs5: NADH dehydrogenase (ubiquinone) Fe-S protein 5; Ndufv2: NADH dehydrogenase (ubiquinone) flavoprotein 2; Neb: EST nebulin; Nudt4: EST Nudix (nucleoside diphosphate linked moiety X)-type motif 4; Nrd1: Nardilysin, N-arginine dibasic convertase, NRD convertase 1; Ostn: osteocrin; Pgls: 6-phosphogluconolactonase; Pgk1: phosphoglycerate kinase; Pgm2: phosphoglucomutase 2; Plekhb1: pleckstrin homology domain containing, family B (evectins) member 1; Pnrc1: proline-rich nuclear receptor coactivator 1; Psmd6: proteasome (prosome, macropain) 26S subunit, non-ATPase, 6; Pygm: muscle glycogen phosphorylase; Rab10: RAB10, member RAS oncogene family; Rp: ribosomal protein; Ryr1: ryanodine receptor 1; Serpinb6a: receptor (TNFRSF)-interacting serine-threonine kinase 1; Slc25a4: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4; Sod1: superoxide dismutase 1, soluble; Sparc: secreted acidic cysteine rich glycoprotein; Srl: sarcalumenin; Sucla2: Succinate-Coenzyme A ligase, ADP-forming, beta subunit; Supt6h: suppressor of Ty 6 homolog (*S. cerevisiae*); Tnnc2: troponin C2, fast; Trim54: tripartite motif-containing 54; Ttn: titin; Ube2b: EST RIKEN cDNA 5430406J06 gene; Ube2g2: ubiquitin-conjugating enzyme E2G 2; Vcp: valosin containing protein; Vdac3: voltage-dependent anion channel 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

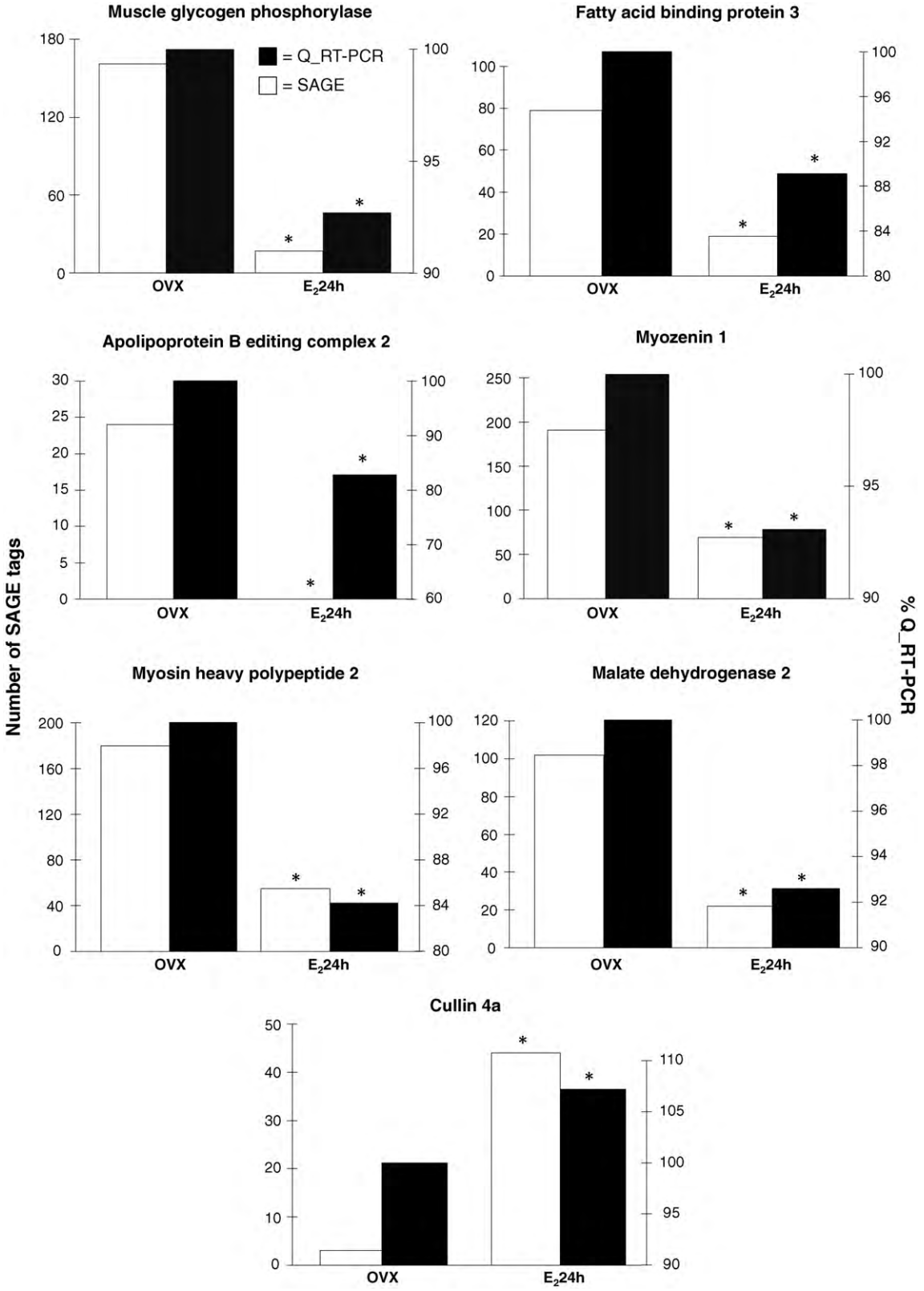

Fig. 3. Confirmation of SAGE results by real-time PCR. *Abbreviations*: E_2: 17β-estradiol; OVX: ovariectomized; Q_RT-PCR: quantitative real-time PCR; SAGE: serial analysis of gene expression. $^*p < 0.05$.

sarcomeric calcineurin, involved in the regulation of genes affecting muscle differentiation and the formation of slow type fibers [41]. On the other hand, the up-regulated ERG cysteine and glycine-rich protein 3 (Csrp3), which is an essential regulator of myogenic differentiation in striated muscle, has also been reported to be induced under conditions leading to transition from fast to slower phenotype in skeletal muscle [42]. In addition, Zhao et al. [43] have shown that sarcalumenin knockout mice exhibit altered store-operated

Ca^{2+} entry and enhanced muscle fatigue resistance. Sarcalumenin is a Ca^{2+} binding protein and its gene expression was repressed by E_2 in the present study. In general, slow type fibers have a higher muscle fatigue resistance than fast type fibers.

24 h after E_2 injection, 6 ERGs related to differentiation and/or microtubule depolymerization such as BTB (POZ) domain containing 1, catenin beta (Ctnnb1), secreted acidic cysteine rich glycoprotein and tripartite motif-containing 54 were also down-regulated, whereas only 2 transcripts, namely Csrp3 and pleckstrin homology domain containing family B (evectins) member 1, were up-regulated. Moreover, E_2 repressed all the transcripts related to cell proliferation (Ctnnb1, cellular nucleic acid binding protein 1 and janus kinase 1), cell cycle arrest (myeloid leukemia factor 1) and apoptosis (Nur77 downstream gene 2, EST homeodomain interacting protein kinase 3 and valosin containing protein). These results suggest that E_2 injection suppresses cell proliferation, cell cycle arrest, apoptosis and differentiation at that time point.

The present study showed that 12 out of 14 transcripts related to protein synthesis and 4 out of 5 transcripts related to protein degradation were repressed by E_2 administration. One of the induced ERGs, ribosomal protein S15, is known to be induced by immobilization [23]. Moreover, kinase domain of titin senses mechanical load, controls muscle gene expression and protein turnover [44]. These results support the role of titin in protein turnover.

4.3. E_2 effects at 3 h

In contrast to $E_2$24 h, E_2 up-regulated transcripts related to muscle structure proteins, namely EST nebulin, a regulator of thin filament length [45], desmuslin which is involved in maintaining muscle integrity [46] and another isoform of Ttn. Although the main role of titin cited above, it appears that disruption of titin causes impaired sarcomerogenesis and results in thin and poorly contractile muscle cells [47]. Car3 corresponds to an enzyme present in slow skeletal muscle and associated to OxPhos [48]. The down-regulation of this transcript at 3 h supports the idea that E_2 injection favors induction of fast-type related transcripts. E_2 also induced transcripts related to cell proliferation/growth (cardiomyopathy associated 5, cold shock domain protein A) and apoptosis (Cul4a). In addition, Ca^{2+}-related transcripts (calmodulin 1 and ryanodine receptor 1) were elevated at this time point. These results clearly show a different modulation from E_2 on diverse functions related to the structure and growth of skeletal muscle at 3 h and 24 h.

4.4. Metabolism

4.4.1. Sugar metabolism at $E_2$24 h

Insulin-stimulated glucose transport via glucose transporter 4 (GLUT4) in skeletal muscle is the major cellular mechanism for glucose disposal in this insulin-sensitive tissue. Insulin binding to its receptor on the cell membrane leads to the translocation of GLUT4 to the cell membrane, allowing the glucose to enter in the cell. Thus, the concentration of GLUT4 to the cell membrane limits the rate of glucose uptake into muscle cells. In the present study, GLUT4 expression in OVX mice was not affected by E_2 injection, which is concordant with previous studies [49,50]. In the present study, member RAS oncogene family (Rab10) was down-regulated by E_2 at 24 h. Rab10 is a small G-protein that, in its active form, participates to insulin-stimulated GLUT4 translocation in adipocytes through its involvement in vesicle movement and vesicle fusion [51]. However, in muscle cells, it appears that Rab10 does not participate in GLUT4 translocation [52]. Other ERGs of importance at this time point include the down-regulated osteocrin (Ostn), also known as musclin, corresponding to a protein exclusively expressed in skeletal muscle. Its expression is regulated by hormonal factors, such as insulin. In fact, the higher expression level is detected in the skeletal muscle of insulin-resistant mice [53]. Functionally, recombinant musclin significantly attenuates insulin-stimulated glucose uptake and glycogen synthesis in myocytes [53].

Two estrogen receptors (ERs), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), are expressed in skeletal muscle [54,55]. ERα is the major ER expressed in this tissue [55]. These nuclear hormone receptors are transcription factors that act by regulating transcriptional processes. Barros et al. [54] suggested that in cells expressing both ERα and ERβ, the receptors oppose each other's action and the ratio of ERα to ERβ determines what type of action E_2 will elicit. Additionally, they have demonstrated that a reduced GLUT4 expression in the skeletal muscle of ERα knockout mice could contribute to insulin resistance, suggesting a repressive role of ERβ on GLUT4 expression [54]. Proline-rich nuclear receptor coactivator 1 (Pnrc1), a nuclear receptor coactivator interacting with ERα [56], is down-regulated at 24 h. Interestingly, this time point showed the highest number of repressed ERGs in the present study.

Three glycogenolysis- and glycolysis-related transcripts were down-regulated, including Pygm, which is responsible of the rate-limiting step of glycogenolysis. On the other hand, two up-regulated ERGs, 6-phosphogluconolactonase (Pgls) and EST aldolase 1 A isoform (Aldoa), were induced. These transcripts code for enzymes involved in the pentose-phosphate pathway (PPP), the alternative route for the metabolism of glucose. The product of this up-regulated pathway, nicotinamide adenine dinucleotide phosphate (NADPH), is produced during the oxidative phase and is involved in protection against the toxicity of reactive oxygen species (ROS). Interestingly, at the same time point, superoxide dismutase 1 (Sod1), encoding for an enzymatic protein protecting blood cells from oxidative stress and damage by destroying free superoxide radicals, was down-regulated. In Sod1 knockout mice, animals show elevated oxidative stress [57,58]. Sod1 knockout mice also directly show loss of muscle mass, similar to the pattern of muscle loss seen in wild-type mice during normal aging [57], as well as a significant decrease in average and maximum life span [58]. Moreover, antioxidant activity of glutathione transferase in satellite cells derived from the elderly is drastically reduced compared to the cells isolated from young individuals [59]. In the present study, we report a down-regulation of glutathione S-transferase theta 1 (Gstt1). The late down-regulation of these two transcripts by E_2, also present at $E_2$6 h, appears to favor an increase of oxidative damage.

4.4.2. Glucose metabolism at $E_2$3 h

$E_2$3 h shows a similar expression pattern to $E_2$18 h. In the latter time point, Prkcsh was late up-regulated, suggesting a positive regulation of glucose uptake at this time point. Involved in GLUT4 vesicle trafficking, Prkcsh has been shown to enhance glucose transport through GLUT4 [29]. Moreover, Glycam1, corresponding to a glycoprotein elevated in diabetic mice, is down-regulated at $E_2$3 h. Diabetic endothelial damage is linked with altered insulin signaling [60], thus suggesting that the down-regulation of this transcript favors a positive regulation of insulin signaling.

4.4.3. Lipid metabolism and RNA metabolism

ERGs related to lipid metabolism were down-regulated in $E_2$24 h, namely enoyl coenzyme A hydratase 1 peroxisomal (Ech1) and Fabp3. Ech1 is coding for a peroxisomal enzyme involved in the fatty acids beta-oxidation pathway. Fabp3 enhances the transport of fatty acids from cell membrane to the site of oxidation. Type II diabetic patients show reduced rate of lipid oxidation by muscle and impairment of the capacity to metabolize free fatty acids, which lead to insulin resistance of skeletal muscle [61]. The down-regulation of those transcripts suggests a decreased

oxidative degradation of lipids through the impairment of free fatty acids transport.

From the AID/APOBEC family, Apobec2 was down-regulated at 6 h. It was proposed that the APOBEC2 gene was first functionally related to RNA editing, by analogy with APOBEC1 [62]. Up to now, APOBEC2 has been detected in skeletal and cardiac muscle, but its main function remains unknown. However, a study in knockout mice demonstrated that there was no obvious effect of APOBEC2 deficiency on mouse health, fertility, or survival up to 1 year of age [63].

4.4.4. TCA cycle, OxPhos and ATP synthesis

Transcripts related to OxPhos and ATP synthesis were up- and down-regulated at 3 h and 24 h, respectively. Those related to TCA cycle and amino acid metabolism were down-regulated at $E_2$6 h and $E_2$24 h. These results are concordant with the repression of lipid metabolism-related transcripts previously seen at $E_2$24 h. Equally, these results do not favor an oxidative metabolism. The impairment of ATP production causes cellular dysfunctions, especially in tissues with high-energy expenditure such as the heart and skeletal muscle. This impairment plays a role in a large selection of pathological processes including myopathy, obesity, and insulin resistance [64]. OxPhos is also the major endogenous source of ROS generation, the toxic by-products of respiration [64].

5. Conclusion

In conclusion, our investigation of the transcriptional changes in skeletal muscle at different time points in response to a single physiological E_2 injection revealed important and distinct changes in modulation at 3 h and 18 h versus 6 h and 24 h. Two categories of modulated transcripts predominated (Fig. 1): those related to skeletal muscle structure and growth and those related to metabolic function. Moreover, changes in gene expression of ERGs related to skeletal muscle structure and growth were concomitant to those reported in the expression of metabolism-related ERGs in this tissue.

At $E_2$6 h and in a more apparent pattern at $E_2$24 h, E_2 injection down-regulated ERGs related to glycolysis, lipid metabolism, OxPhos and ATP synthesis, TCA cycle and protein metabolism. This was reflected in the reduced expression of ERGs linked to fast type skeletal muscle and to skeletal muscle structural proteins. This is also supported by the down-regulation of transcripts involved in cell growth/proliferation and cell differentiation at the same time point. At $E_2$18 h and in a more apparent pattern at $E_2$3 h, the E_2 injection up-regulated ERGs associated to OxPhos, ATP synthesis, cell signaling and growth/proliferation.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.12.004.

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