Biochemical and Biophysical Research Communications 421 (2012) 736–742

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Validation of differential gene expression in muscle engineered from rat groin adipose tissue by quantitative real-time PCR

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ARTICLE INFO

Article history:
Received 3 April 2012
Available online 20 April 2012

Keywords:
Reference genes
Data normalization
Engineered skeletal muscle
Quantitative real-time PCR
Muscle tissue engineering

ABSTRACT

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a highly sensitive tool that can be used for accurate and reliable gene expression analysis; however, a critical factor for creating reliable data in relative quantification is the normalization of the expression data of the genes of interest. In this study, we demonstrate the important process of validating four muscle-specific genes (myosin, desmin, MEF2D and ADAM12) and 10 common potential reference genes (β-2-microglobulin, RPL32, RPL17, α-tubulin, CYC, ET1A, β-actin, HSPCB, SDHA and GAPDH) in engineered muscle tissues. Tissue samples were generated out of rat groin adipose tissues by myogenic induction in a perfusion bioreactor for 7, 21 and 49 days. Results of analyzed muscle-specific genes suggested that the gene expression pattern corresponding to myogenic induction observed in adequately treated rat adipose tissue was time-dependent, making the length of time in culture in myogenic medium an important factor. Our data suggest that the reference genes were expressed variably in the different samples. During engineered muscle development, β-2-microglobulin, RPL32 and RPL17 were the most stably expressed genes. The commonly used reference genes β-actin and GAPDH appeared to be too unstable for normalization of qRT-PCR expression in engineered muscle tissue. The use of β-2-microglobulin, RPL32 and RPL17 as internal standards may improve the accuracy of gene expression studies aimed at muscle tissue engineering under the proposed settings.

1. Introduction

Gene expression analysis is increasingly important in biological research and can provide a theoretical basis for complex regulatory networks. To study expression changes and compare mRNA levels of single genes, quantitative real-time RT-PCR (qRT-PCR) offers the most sensitive method by measuring the quantity of amplicons in real-time after reverse transcription (RT) of mRNA to complementary DNA (cDNA) [1–3]. qRT-PCR is also fast, easy to use and provides simultaneous measurement of gene expression in many different samples for a limited number of genes [4,5].

The selection of appropriate reference genes for data normalization is a critical step in the process of obtaining accurate and reliable gene expression profiles, especially when the expression differences are quite small or when the samples are from different histological origins or stages of development [6]. Various normalization strategies have been suggested to help decrease the variability observed in qRT-PCR, including the use of internal standard reference genes, normalization to starting material quantity, and the use of external control nucleotides of known quantity [7,8]. While there are pitfalls to all normalization approaches, the use of internal reference genes has proven to be most accepted because these endogenous genes are expressed and amplified under the same experimental conditions as the genes of interest [9,10]. However, recent studies have indicated that the most commonly used reference genes, such as β-actin and GAPDH, can not be relied upon to be stably expressed under all experimental conditions [11].

In recent years, increasing trends became apparent at muscle tissue engineering aiming at the reconstruction of skeletal muscle loss. In our research, we focus on creating efficiently large volumes of vascularized muscle tissue by using rat groin adipose tissue in perfusion bioreactors. However, to date there has not been an extensive statistical analysis of reference gene stability in engineered skeletal muscle tissue. In the present study, we assessed the expression stability of 10 potential reference genes and four rat muscle-specific genes in the engineered skeletal muscle tissue at three different time points (after an incubation period of 7, 21 or 49 days, respectively, in perfusion bioreactors). The 10 reference genes examined were: β-2-microglobulin (B2M), glyceralde-hyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein L17 (RPL17), ribosomal protein L32 (RPL32) succinate dehydrogenase complex, subunit A (SDHA), ET1A (E. tenella refractile body gene), HSPCB (Heat Shock Protein 1, beta), CYC (Cyclophosphamide), α-TUB (α-tubulin), β-actin (ACTB). The four muscle specific

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http://dx.doi.org/10.1016/j.bbrc.2012.04.073

genes examined were myosin, desmin, MEF2D and ADAM12, respectively.

2. Materials and methods

2.1. Experimental design

A total of 24 adult male Lewis rats of 250–290 g were used in this study. They were used to elevate adipofascial flaps based on the epigastric vessels, a branch of the femoral vessels. The flaps consisted of fat tissue and fascia but not skin. The technique was conducted according to the current regulations and principles of the German law for animal welfare. Under isoflurane anesthesia using an animal narcotic unit (Euthanex Isoflurane), the groins of rats were shaved with an electrical shaver and followed by disinfection of the operative site with octenisept (Schülke & Mayr GmbH, Germany). A 2 cm skin incision was made in the bilateral groin parallel to and adjoining the inguinal ligament. The inguinal adipofascial flaps was exposed and a blunt tipped needle was inserted into the dissected femoral artery. Subsequently, the elevated flap was placed in the perfusion bioreactors. The rat was humanely killed under anesthesia by transecting the heart. Myogenic differentiation was assessed on days 7, 21 or 49 by qRT-PCR.

2.2. Tissue and total RNA preparation

At 7, 21 or 49 days after incubating in perfusion bioreactors, the engineered muscle samples (approximate 100 mg) were cut into several pieces, and immediately frozen in liquid nitrogen.

Total RNA was isolated from the tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and NucleoSpin RNAII Kit (MN Macherey–Nagel, Dueren, Germany) according to the manufacturers' protocol. The RNA concentration was measured by photometry at 260 nm (Nanodrop, Peqlab, Erlangen, Germany). RNA purity was determined by measuring 260/280 and 260/230 ratios. Furthermore, the quality of the total RNA was verified by assessing the integrity of 18S/28S ribosomal RNA in 1% ethidium bromide-stained agarose gels (Biozym, Hessisch Oldendorf, Germany).

2.3. Muscle specific genes and reference genes design

Four primers for muscle specific genes used in the qRT-PCR reaction were designed based on information from the rat genomic database (NCBI). Furthermore, 10 potential reference genes were chosen based on their common use as endogenous control genes in rat gene expression studies. The sequences, length of products, and source sequences are listed in Table 1. The assays setting "SYBR Green Design" were chosen to limit primer sequences to regions with little secondary template structure. The designed primer sequences were validated by BLAST in order to ensure high binding efficiency.

2.4. cDNA synthesis and quantitative real-time PCR (qPCR)

Reverse transcription (RT) was performed with 1 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA; USA) according to the protocol provided with the kit. Real-time polymerase chain reactions (RT-PCR) were carried out in 20 μl samples with 2.5 ng cDNA and 10 pmol of each forward and reverse primer using the 2xSsoFast EvaGreen Supermix Kit (Bio-Rad Laboratories).

The initial denaturation step at 95 °C for 10 s was followed by 40 cycles of denaturation for 10 s at 95 °C, annealing and extension for 30 s at 60 °C. Melt curve data were collected starting at 70 °C increasing temperature at 0.2 increments by 125 cycles for 15 s. The PCR products were fractionated and visualized on 2% agarose gels containing ethidium bromide. All of the experiments were carried out in triplicates and repeated at least at three independent times. The specificity of the RT-PCR products was proved using the appropriate melting curves (specific melting temperature) and product size evaluation on 2% agarose gels supplemented with ethidium bromide. The mean quantification cycle (Cq) value was used for further analysis.

2.5. Analysis of gene expression data

RT-PCR data were exported into an Excel datasheet and analyzed using reference gene stability analysis software program qBase [12]. qBase is an Excel-based tool for the management and automatic analysis of real-time quantitative PCR data. The current consensus is that multiple stably expressed reference genes are required for accurate and robust normalization, especially for measuring subtle expression differences. While different tools are available to determine which candidate reference genes are stably expressed (for example, geNorm [13], BestKeeper [14], Normfinder [15]), almost no software is available to perform straightforward normalization using more than one reference gene. qBase allows gene expression levels to be normalized reference genes that can easily be selected from the gene list. qBase software was used accordingly to calculate normalized relative quantities expressed as arbitrary units of gene expression.

3. Results

3.1. RNA quantity and quality

It is well known that RNA quality and quantity are critical for successful gene expression analysis [16]. This requires quantification of the isolated RNA as well as assessment of the RNA integrity. In this study, RNA from different experimental conditions was quantified by photometry, showing that RNA purity, which was determined by measuring 260/280 and 260/230 ratios, was very high. Intact rRNA subunits of 18S and 28S were observed on the gel electrophoresis, indicating that the degradation of the RNA was minimal.

3.2. Expression of reference genes of engineered muscle tissue

The dissociation curves of the 10 potential reference genes exhibited signal cusps, and the dissociation temperatures were all greater than 80 °C. In addition, qRT-PCR analysis of every gene was performed in triplicate for each of the 5 cDNAs, along with no template and RT-minus controls. The 10 primer pairs used to amplify the candidate reference genes generated single amplicons of the expected size from the various cDNA pools, as shown by the presence of single bands in agarose gel electrophoresis (Fig. 1A).

3.3. Determination of the stability of reference genes and muscle specific genes by qBase

The qBase program is an Excel-based tool that provides a measure of gene expression stability (*M* values). An *M* value is the mean pair-wise variation between an individual gene and the other putative reference genes tested [13]. Genes with lower *M* values have more consistent expression patterns. From the transformed data, qBase produced a graph based on expression stability values (*M* values) (Fig. 1B). When all samples were analyzed as one data set, the *M* value of β-2-microglobulin was the lowest, whereas the *M* value of GAPDH was the highest. This indicates that β-2-microglobulin was the most consistently expressed

Table 1
Reference genes and muscle-specific genes used for real-time quantitative PCR.

Gene symbol	Strand	Nucleotide sequence	Size of PCR products (bp)
β-Actin	Sense	5′-CAGAGCAAGAGAGGCATCCTC-3′	363
	Antisense	5′-GTCCAGACGCAGGATGGCATG-3′	
GAPDH	Sense	5′-AGACAGCCGCATCTTCTTGT-3′	239
	Antisense	5′-TTCCCATTCTCAGCCTTGAC-3′	
RPL32	Sense	5′-AGATTCAAGGGCCAGATCCT-3′	196
	Antisense	5′-CTACGAAGGCTTTTCGGTTC-3′	
RPL17	Sense	5′-CCAGGTGAACAAGGCTCCTA-3′	208
	Antisense	5′-AATTTATTCCCGTGCCATGA-3′	
SDHA	Sense	5′-CGAGATCCGTGAAGGAAGAG-3′	187
	Antisense	5′-GCCCATGTTGTAATGCACAG-3′	
α-tubulin	Sense	5′-GCTTCTTGGTTTTCCACAGC-3′	162
	Antisense	5′-TGGAATTGTAGGGCTCAACC-3′	
ET1A	Sense	5′-GGGTCCTGGACAAACTGAAA-3′	187
	Antisense	5′-ACACCAGCAGCAACAATCAG-3′	
β-2-microglobulin	Sense	5′-TGACCGTGATCTTTCTGGTG-3′	151
	Antisense	5′-ATCTGAGGTGGGTGGAACTG-3′	
CYC	Sense	5′-CCCACCGTGTTCTTCGACAT-3′	116
	Antisense	5′-CCAGTGCTCAGAGCACGAAA-3′	
HSPCB	Sense	5′-GATTGACATCATCCCCAACC-3′	247
	Antisense	5′-CTGCTCATCATCGTTGTGCT-3′	
Desmin	Sense	5′-ACCTGCGAGATTGATGCTCT-3′	206
	Antisense	5′-ACATCCAAGGCCATCTTCAC-3′	
ADAM12	Sense	5′-GGAAAGGCCCACTGATGAG-3′	98
	Antisense	5′-ATTGAGGGGCCTGCTGATG-3′	
MEF2D	Sense	5′-GACATGGACAAGGTGCTGCTCAAG-3′	127
	Antisense	5′-CATCCGGCTCTGGGCTGTCA-3′	
Myosin heavy chain	Sense	5′-CTACTCGGGCCTCTTCTGTG-3′	203
	Antisense	5′-TTCCAGCTCCAGACTCACCT-3′	

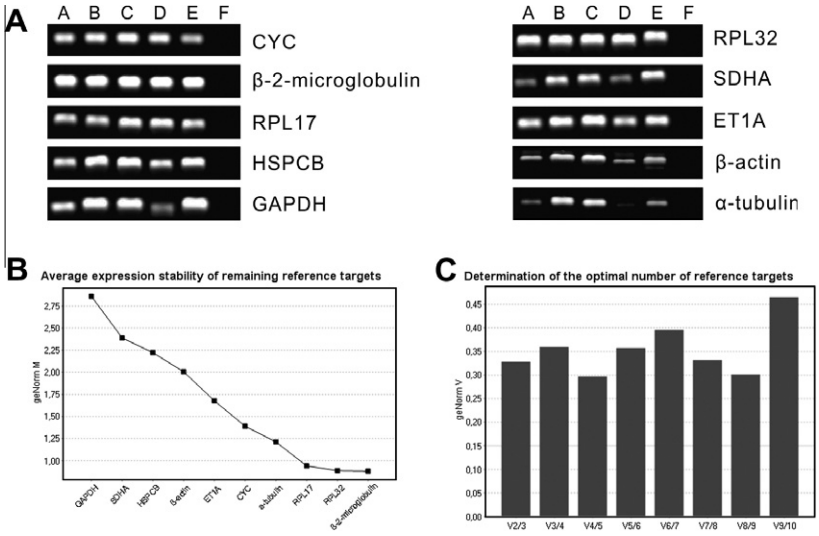

Fig. 1. (A) Specificity of qRT-PCR amplification. Agarose gel (2%) showing amplification of a specific PCR product of the expected size of 10 reference genes in engineered muscle tissue after in vitro culturing for 7 days (A), 21 days (B), 49 days (C), groin adipose tissue (D), native muscle (E) and no template control (F). (B) Average expression stability values and ranking of reference genes assessed by qBase. The graph represents the stability values (*M*, *y*-axis) of 10 reference genes and the associated ranking from least to most stable expression (*x*-axis). Lower *M* value indicates more stable gene expression. (C) Determination of the optimal number of reference genes for normalization assessed by qBase. Each bar represents the pairwise variation (*V*) between two sequential normalization factors containing an increasing number of genes. The cut-off value is set as 0.15.

gene, followed by (from most to least stable) RPL32, RPL17, α-tubulin, CYC, ET1A, β-actin, HSPCB, SDHA and GAPDH. The qBase analysis demonstrated that GAPDH was the most variably expressed gene in all of the sample sets.

A single reference gene alone is not recommended as an appropriate internal control in the experiment. Therefore, two or more reference genes are necessary for accurate normalization. qBase determines the number of reference genes necessary for normalization by calculating the average pairwise variation (*V*) value. By adding each gene in the calculation of the normalization factor (NFn) in order to their increasing *M* values (decreasing stability) the effect of each reference gene included is reflected by a changing variance *Vn*/*n* + 1 of the NF*n* + 1. It is proposed to use a cut-off value for the pairwise variation of 0.15, below which the inclusion of an additional reference gene is not required [13]. As shown in (Fig. 1C), when all samples were considered as a single data set, the pairwise variations *V*2/3, *V*3/4, *V*4/5, *V*5/6, *V*6/7, *V*7/8, *V*8/9 and *V*9/10 were all higher than the cut-off value of 0.15, indicating that the addition of a 10th reference gene for normalization would be necessary or put otherwise none of the analyzed housekeeping

Table 2
The *M* values of each candidate reference gene.

	7 Days	21 Days	49 Days	Fat	Muscle
CYC	0.393	4.151	7.396	0.751	0.11
ET1A	0.169	6.205	16	0.095	0.63
GAPDH	0.041	4.891	62.106	0.002	32.9
HSPCB	0.107	5.802	10.828	0.031	4.879
RPL17	0.82	0.942	1.602	1.427	0.566
RPL32	0.701	1.936	2.555	0.441	0.654
SDHA	0.076	2.214	13.423	0.043	10.411
a-Tubulin	0.36	5.134	4.267	0.232	0.546
β-2-Microglobulin	1.074	2.149	2.412	0.631	0.285
β-Actin	0.105	10.411	29.446	0.036	0.859
Normalization factor	0.235 ± 0.008	3.573 ± 0.170	8.17 ± 1.425	0.122 ± 0.012	1191 ± 0130

genes was stably expressed among the samples. This was an unexpected finding in a collection of samples derived from fat tissue kept in a perfusion bioreactor under myogenic conditions over different time periods compared to native fat and muscle tissue.

In our previous studies we could show that fat tissue, when perfused continuously with muscle inducing agents, is able to adapt muscle-like features in histological analysis. So we next analyzed normalized gene expression in our bioreactor samples in detail. In addition, when the data shown in Table 2 were displayed respectively in a bar chart diagram, it became that clear expression patterns of the engineered muscle tissue at 7 days is more similar to that of fat and at later time points is more similar to that of the muscle which is especially the case at 49 days (Fig. 2). Regarding the reference genes, we found high expression values for RPL32, RPL17, α-tubulin, β-2-microglobulin, and CYC in fat and tissue kept in the bioreactor for 7 days, while observing the opposite phenomenon for SDHA, GAPDH, β-actin, HSPCB, which were highly expressed in bioreactor tissue at 49 days and muscle but showed low expression values in fat and bioreactor tissue at 7 days. It demonstrated that bar chart of muscle differentiation at 7 days is similar to that of adipose tissue, and increase with time, and at 49 days the bar chart of engineered muscle is similar to the one of native skeletal muscle (Fig. 3).

Consistently, the expression patterns of four selected muscle-specific genes (Fig. 4), showed a similar trend. The genes expressed at 7 days in bioreactor tissues were still similar to rat groin adipose tissue samples used as controls, with a continuous increase of muscle-specific gene expression over the whole incubation period. The bar chart of specific muscle gene expression of muscle differentiation peaked at 49 days, which is similar to that of native skeletal muscle.

4. Discussion

qRT-PCR has become a valuable tool for accurate gene expression profiling. A critical step for an accurate analysis is to minimize the sample and experimental variation by normalizing the raw expression data to the expression of a reference gene. Gene expression analysis is the most common strategy applied to identify the genes relevant to new signaling and metabolic pathways that underlie many biological processes. Consequently, it is critical to select appropriate reference genes for data normalization in gene expression analysis. Ideally, reference genes should show consistent expression levels in all cell types and tissues under study, and transcription should be constant relative to global cellular transcription rates under different experimental conditions [17]. Nevertheless, evidence has shown that expression of individual reference genes does differ among samples under different experimental conditions. Therefore, it is essential to select a suitable reference gene for normalization in the according experimental setting. To evaluate 10 reference genes for expression normalization for engineered muscle tissue, we used qRT-PCR to examine mRNA expression of these genes and applied qBase methods to evaluate the expression consistency in the five sample sets as described above.

Historically, the most frequently used reference genes were β-actin and GAPDH [18], as they were used for many years in Northern blots, RNase protection assays, and later, in conventional RT-PCR. However, recent studies have indicated that these common reference genes are not stably expressed under all experimental conditions [19]. In the present study, among the 10 candidate reference genes evaluated, β-2-microglobulin, RPL32 and RPL17 were the most stably expressed in rat engineered muscle, whereas GAPDH was the least stable. Some studies have been done in rat skeletal muscle tissue using qPCR. Yüzbaşioğlu et al. [20] suggested that the TATA box binding protein (TBP) and the β-actin gene may be suitable reference genes in gene expression studies of rat soleus and gastrocnemius muscle with chronic degenerative changes. Sun et al. [19] selected RPL13 and RPL32 as the ideal reference genes for accurate normalization in contused rat skeletal muscle. In human myoblast studies, Stern-Straeter et al. [21] proposed TATA box binding protein and large ribosomal protein P0 (RPLPO) as reference genes for normalization of gene expression studies during the differentiation of human myoblasts. However, few studies have compared the stability of reference genes of engineered muscle tissue. In our study, we sought to produce an engineered, vascularized skeletal muscle construct with an axial vascular supply for the first time and compared the stability of reference genes of large volume engineered muscle constructs. Interestingly, β-actin and GAPDH were also found to have a highly variable expression in rat engineered muscle and were deemed unsuitable for normalization of qRT-PCR data. They were both rejected by the qBase program due to high *M* values compared to other candidates. Therefore, the GAPDH and β-actin genes were not suitable as internal controls for muscle tissue engineering, due to their highly variable expression.

In recent years, the use of a single reference gene for data normalization has been discouraged in light of variations in reference gene expression and resultant misinterpretation of expression data [22]. Some authors have proposed that at least three well-validated housekeeping genes should be used to normalize expression data. In our study, according to qBase analysis, when all samples were considered together, β-2-microglobulin, RPL32 and RPL17 were the most consistently expressed genes. Similarly, from agarose gel electrophoresis analysis, the band intensities in these three genes were stronger than the other seven reference genes observed in all samples. β-2-microglobulin (B2M) encodes a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells. Klein et al. [23] also identified B2M as the most stably expressed gene in equine endometrial, testicular, and conceptus tissues. Ribosomal proteins RPL32 and RPL17 are both components of the 60S ribosomal subunit. Although several ribosomal proteins are variably expressed under disease conditions

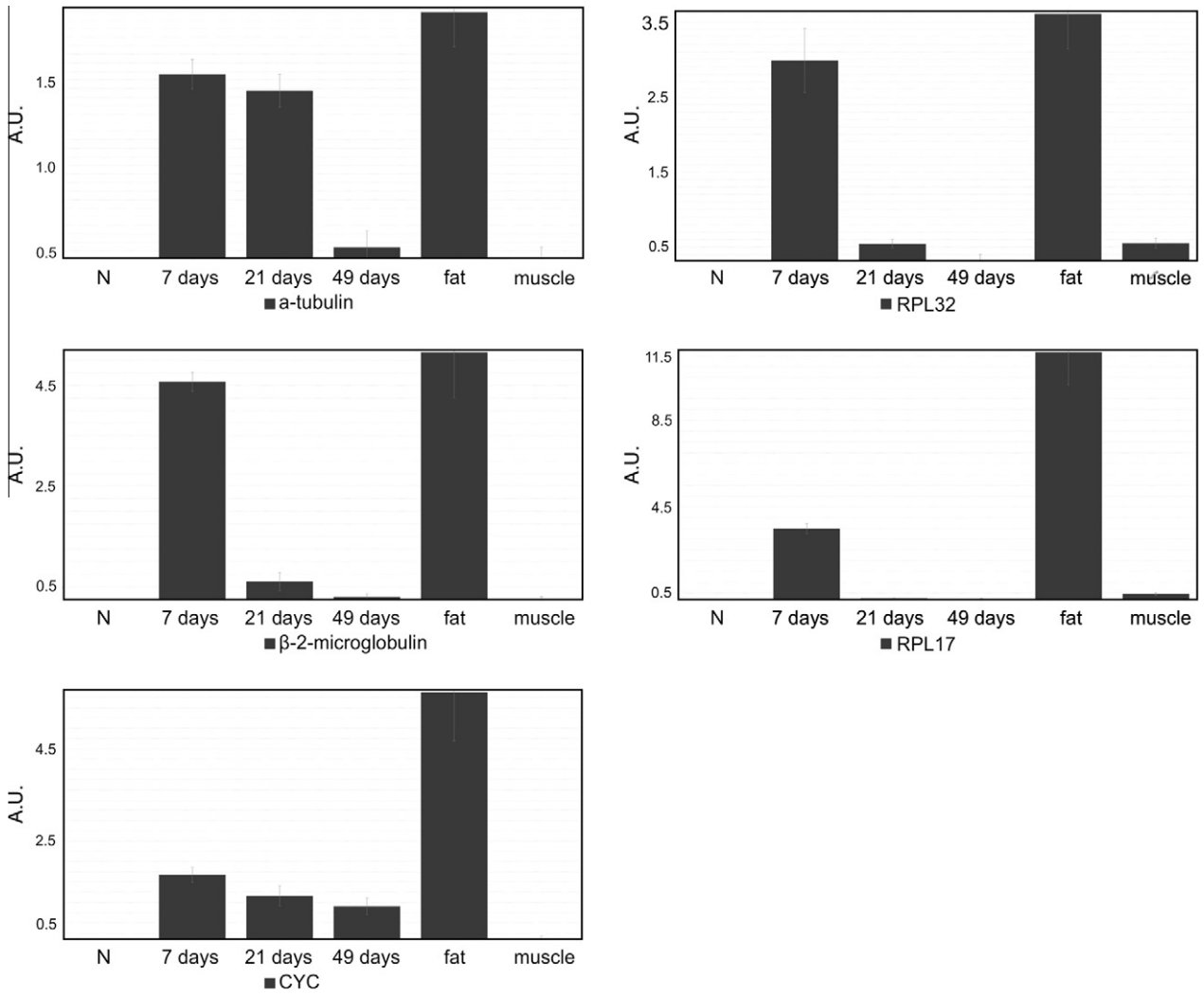

Fig. 2. Similarity in the expression profile of five reference genes in engineered muscle tissue originating from rat groin fat tissue at 7 days, 21 days or 49 days. Bars indicate normalized relative quantities expressed in arbitrary units (A.U.).

(e.g. gastrointestinal cancer) [24], they have also been used as normalization genes [19].

Moreover, qBase could also be used to determine the number of reference genes necessary for normalization by calculating the average pairwise variation (V) value. When the V value is below 0.15, the inclusion of an additional reference gene is not required. When all samples were considered together, the $Vn/n+1$ were all >0.15, indicating that among the chosen set of genes none was expressed stably enough to serve as a reliable normalization factor. Analysis of other genes, which might be expressed more stably, or addition of a 10th reference gene for normalization is necessary.

Generally, the expression pattern of bioreactor tissues kept for 7 days in the perfusion bioreactor was similar to rat groin adipose tissue samples used as controls, while a continuous increase of muscle-specific gene expression was observed over the whole incubation period. Results of analyzed muscle-specific genes suggested that the gene expression of myogenic factors in perfused rat adipose tissue was time-dependent, making the length of time in culture in myogenic medium an important factor. These findings indicate that a transdifferentiation is going on in the perfused adipose flaps. As can be seen on gene level, large volumes of vascularized adipose tissues transdifferentiated to muscle-like tissues when cultured in perfusion bioreactors.

In conclusion, in this study, we investigated for the first time the most reliable house keeping genes for the normalization of real-time qRT-PCR data in rat engineered muscle tissues, using qBase program. Although there is a limitation of using an animal model for estimating large volumes of engineered muscle tissues, and the data obtained on the basis of this animal model might not be fully valid for human material, the use of an animal model system seems justified in the present situation. This study analyzed a set of reference genes for normalization of gene expression profiles in large volumes of engineered muscle tissue using qRT-PCR. Though we saw surprisingly high variations in expression, β-2-microglobulin, RPL32 and RPL17 emerged as the most appropriate reference genes following rat engineered muscle tissue, while the commonly used reference genes β-actin and GAPDH appeared to be too unstable under this experimental design. Thus, the identified pairs of stable reference genes may be used for future expression studies to help to determine engineered muscle in regeneration medicine. This study further emphasizes the need to accurately validate candidate reference genes in the model under study before employing them in gene expression studies using qRT-PCR. In addition, the gene expression of myogenic factors in perfused rat adipose tissue was time-dependent, making the length of time in culture in myogenic medium an important factor.

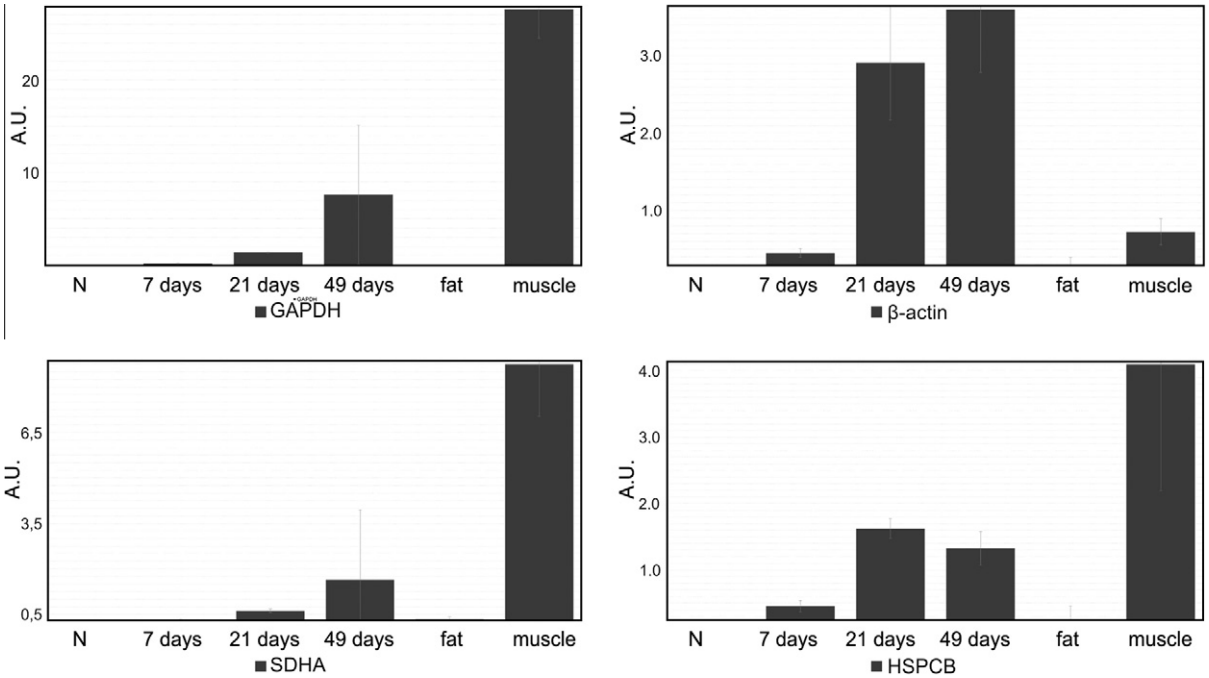

Fig. 3. Distribution of the similar trend of expression of four reference genes in engineered muscle tissue from rat groin fat tissue at 7 days, 21 days or 49 days.

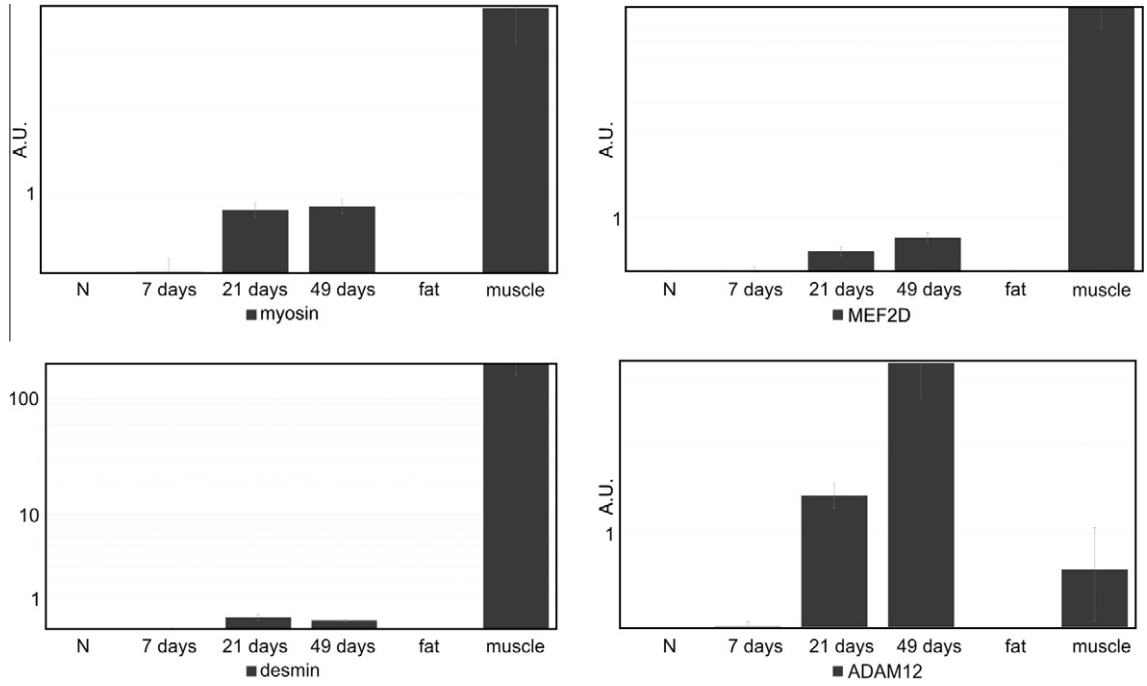

Fig. 4. Distribution of a continuous increase in expression of four muscle-specific genes in engineered muscle tissue originating from rat adipose fat tissue at 7 days, 21 days or 49 days. No expression of the chosen muscle-specific genes could be detected in rat groin fat tissue samples used as controls.

We further confirmed on gene level by qBase analysis that large volumes of vascularized adipose tissues could be transdifferentiated to muscle-like tissues, when cultured in perfusion bioreactors.

Acknowledgments

The authors want to thank Vesna Bucan and Stefanie Michael for helpful comments on the manuscript.

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