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Quantitative monitoring of the mRNA expression pattern of the TGF-β-isoforms (β1, β2, β3) during transdifferentiation of hepatic stellate cells using a newly developed real-time SYBR Green PCR

L. Wickert,* S. Steinkrüger, M. Abiaka, U. Bolkenius, O. Purps, C. Schnabel, and A.M. Gressner

Institute of Clinical Chemistry and Pathobiochemistry, RWTH-University Hospital Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany

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Abstract

Current methods to determine the mRNA of the TGF-β-isoforms, β1, β2, and β3, are not sensitive enough to detect small alterations in the expression levels. Therefore, we established a SYBR Green I-based real-time quantitative PCR procedure with fragment-specific standards. The advantage of gene-specific quantification is the possibility to be abstain from the need to compare results with a house-keeping gene having a different sequence and PCR efficiency. Reproducibility of the results and analytical variances of the real-time PCR assays were tested. In transdifferentiating rat hepatic stellate cells (HSC) the TGF-β1-mRNA was found to be the predominant isoform expressed followed by TGF-β3 and low amounts of TGF-β2-mRNA. An alteration of the TGF-β1,-β2, and -β3 ratio during HSC transdifferentiation could not be detected. Furthermore, the GAPDH mRNA expression varied during HSC activation, and thus is not recommended as a standard in real-time PCR quantifications.

Keywords: TGF-β-isoforms; mRNA-quantification; Real-time PCR; Hepatic stellate cells

The TGF-β-isoforms, β1, β2, and β3, have overlapping but also distinct functions in cell growth and differentiation. TGF-β signaling leads to the production of extracellular matrix proteins by activated hepatic stellate cells (HSC) which promote the development of liver fibrosis [1–3]. The spontaneous activation of HSC in culture mimics cell activation in inflamed liver tissue and therefore allows to study in vitro signal transduction of TGF-β and gene expression during transdifferentiation to myofibroblasts (MFB; [4,5]). An important question concerns the mRNA expression patterns of the different TGF-β-isoforms. Several groups quantified the TGF-β-isoforms in hepatocytes, Kupffer Cells, and HSC obtained from normal and fibrotic liver using Northern blot, in situ hybridization, and RNase protection assay. Frequently, RT-PCR is used, followed by gene-specific cDNA detection on agarose gels stained with ethidium bromide [6–12]. By nature, mRNA measurement in the plateau phase of PCR is not suited for quantitative analyses. Therefore, we developed specific assays for mRNA quantification of the rat TGF-β-isoforms β1, β2, β3, and GAPDH. For this purpose real-time-assay procedures were designed as two-step assays using the LightCycler FastStart DNA Master SYBR Green I kit from Roche Diagnostics GmbH.

It is found that during transdifferentiation of HSC to MFB in culture, none of the three TGF-β-isoforms were strongly modulated in their gene expression. The TGF-β1-mRNA was the predominant isoform in most samples followed by the TGF-β3-isoform. TGF-β2-mRNA was found in low quantities. Probably the TGF-β1 and TGF-β3-isoforms are involved in biochemical and morphological regulation during transdifferentiation from HSC to MFB but the functional relevance of the small TGFβ2 proportion has to be elucidated.

Moreover, the GAPDH mRNA transcripts rise during HSC activation and are regulated showing two

* Corresponding author. Fax: +49-241-80-825125.
E-mail address: wickert@unicum.de (L. Wickert).

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peaks of expression during transdifferentiation to MFB. This implies that GAPDH is not suited for use as an internal standard in real-time PCR procedure.

Materials and methods

Cell culture. HSC were prepared from the livers of male Sprague–Dawley rats [13,14]. The cells were seeded in 90 mm culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4 mmol/L L-glutamine, 10% fetal calf serum (FCS), and penicillin (100 IU/ml)/streptomycin (100 μg/ml). The medium was changed approximately 20 h after seeding, and subsequently all 3–4 days. The cell cultures were maintained at 37 °C, 5% CO_2 in a humidified atmosphere. To generate MFB some cell dishes were trypsinized after 7 days.

Isolation of total RNA. Total RNA was collected from HSC and MFB at different time points of culture. According to the manufacturer's instruction RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). To avoid contamination with genomic DNA, RNA samples were treated with RNase-free DNAse I (Roche Diagnostics GmbH, Mannheim, Germany). Dependent on the cell differentiation status the yield of total RNA from about 2.5×10^6 cells varied between 10 and 30 μg. RNA purity was verified in a formaldehyde–agarose gel.

Generation of gene-specific real-time PCR standards. With gene-specific primers a cDNA fragment was amplified and column-purified using the QIAquick PCR purification kit (Qiagen). Following primer sequences were used: rTGFβ1 (GI: 57341) F (TGGCGTTACC TTGGTAACC), R (GGTGTTGAGCCCTTTCCAG); rTGFβ2 (GI: 311324) F(ATCGATGGCACCTCCACATATG), R (GCGAAGG CAGCAATTATGCTG); rTG Fβ3 (GI: 31132) F (AAGCGCACAG AGCAGAGAA TC), R (AGTGTCAGTGACATCGAAG); rGAP DH (GI: 10190788) F (AGCCCAGAACATCATCCCTG), R (CAC CACC TTCTTGATGTCA TC). A T7-sequence was suffixed to the 5′-end of each forward primer. Successful amplification and subsequent purification was confirmed by sequencing on a semiautomated sequencer (310A, Applied Biosystems, Foster City, USA). Thereafter, an in vitro RNA transcription was performed using an in vitro transcription kit (Stratagene, La Jolla, USA). After cDNA digestion cRNA was purified with RNeasy kit (Qiagen) and cRNA-concentration was determined. A gene-specific standard curve was generated from serial 10 time logarithmic dilutions of the cRNA by a reverse transcription. By means of this strategy we achieved a comparable PCR kinetic of standard and sample.

Real-time PCR. To generate cDNA from cell culture samples, 1 μg of total RNA was reverse transcribed with random hexamers using expand reverse transcription polymerase (Roche). Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I from Roche Diagnostics GmbH according to manufacturer's protocol. The following LightCycler conditions were used: Initial denaturation at 95 °C for 10′, followed by 45 cycles with denaturation at 95 °C for 10″, annealing at 60 °C for 5″, and elongation at 72 °C for 15″. Quantities of specific mRNA in the sample were measured according to the corresponding gene-specific standard curve.

Proliferation assay. To determine proliferation of rat HSC, 200,000 cells were seeded in 2 ml DMEM/10% FCS medium with a density of 0.09×10^6 cells/3.8 cm^2 per well on 12-well plates. Medium was changed from 10% to 0.5% FCS one day after seeding. Two days later, cells were preincubated for 1 h with various TGF-β1 concentrations in serum-free DMEM. The cells were then supplemented with 0.5% FCS and after 24 h exposed with [^{3}H]thymidine (2 Ci/ml medium; NEN DuPont) for additional 24 h. Thereafter, [^{3}H]thymidine incorporation was measured as described previously by Zerbe and Gressner [15]. Subsequently, cell cultures were analyzed with an enzyme-linked fluorescence (ELF) assay according to the procedure of Wolff et al. [16], which was modified as described by Roth et al. [17].

Northern blot. Total RNA was separated using 1% formaldehyde–agarose gel and blotted onto nylon membrane (Hybond XL, Amersham Pharmacia Biotech, Buckinghamshire, England). cDNA probe for hybridization was prepared by labeling TGF-β1 fragments according to the sequence of Quian et al. [18] with α [^{32}P]dCTP using a random-priming kit (Life Technologies, Paisley, Scotland). Unincorporated nucleotides were removed by ethanol/ammonium acetate precipitation in the presence of excess carrier DNA.

Results and discussion

Standard design and optimization of SYBR Green I-based RNA quantification

To design a two-step SYBR Green I-based real-time PCR method the gene-specific standard fragment has to have the following properties: (i) Optimal fragment length should have 100–300 bp and an extension over an exon-border. (ii) GC or AT-rich and repetitive sequences should be avoided. Despite of the same RNA extraction procedure, samples from different sources (tissue, cell culture) showed different matrix properties. Hence, this should be tested in a parallel dilution with the standards. A flowchart, demonstrating the strategy of creating a sensitive two-step real-time PCR assay (Fig. 1).

Characterization of real-time PCR assays

To assess the coefficient of variation (CV) of distinct RT-PCR procedures two different samples were reverse transcribed 10-times and 9-times. The calculated CV amounts 2.1% ($n = 10$) and 1.9% ($n = 9$), respectively. Moreover, the intra-series imprecision of 17 samples for TGF-β1, 10 samples for TGF-β2, and 10 samples for TGF-β3 real-time PCR amounts 1.8%, 2.1%, and 0.43%, respectively. Additionally, the variance of different test series was examined with the GAPDH-specific real-time mRNA quantification. For this purpose, nine cell culture dishes of identical culture time were subjected to all steps of the procedure; i.e., cell culture and harvesting, RNA-extraction, RT-PCR, and real-time PCR. Coefficients of variation of 1.4% for crossing point and 14.6% for calculated concentration were obtained. It should be noticed that fragment length, and copy number of the template influence the reproducibility of the PCR. The differences found in the CV between crossing point and calculated concentration are confirmed by the results of Betzl et al. [19], who found that SYBR Green I detection of a template with a copy number of 5000 differs in the CV from 0.6%, crossing point to 13.2% calculated concentration.

However, the CV depends on initial mRNA concentrations in the sample. For example five independent

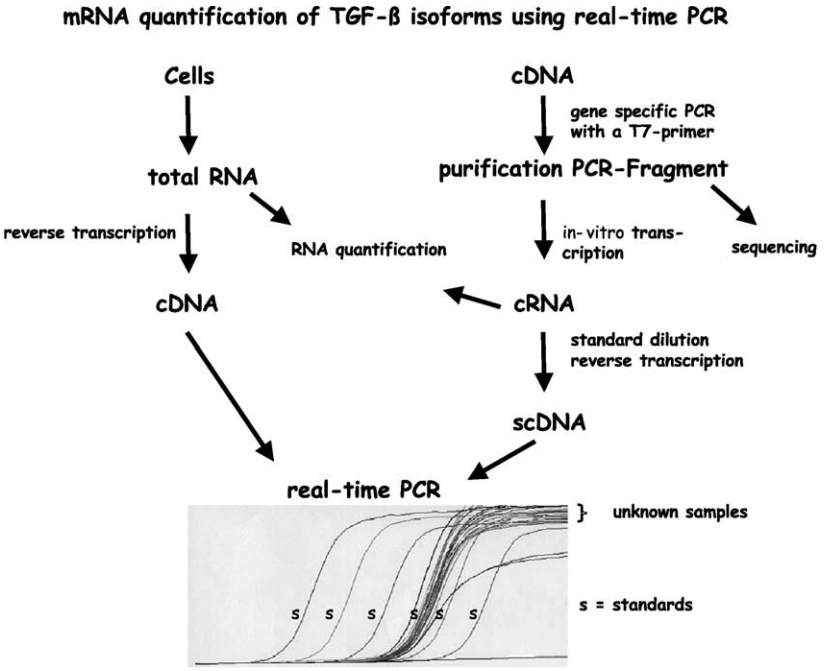

Fig. 1. Flowchart showing the process of creating gene-specific standards and samples for RNA quantification and an example for fluorescence curves of a real-time PCR with standards and unknown samples. Gene-specific standard was generated from serial 10 time logarithmic dilutions of the cRNA by a reverse transcription. LightCycler software creates a standard curve out of the given concentration of each standard versus their crossing points. From this standard curve one can determine concentration of the unknown sample which is expressed as an absolute value.

real-time PCR analyses of TGF-β1 standards which ranged from 700 to 0.007 pg were measured and the CV varied about 1% in the optimized concentration range (data not shown). mRNA concentrations below or above this range lead to elevated CVs.

Expression of the TGF-β-isoforms β1, β2, β3 during HSC transdifferentiation

The distribution of specific immunohistochemical markers characterizes the transdifferentiation stage of culture activated HSC [20]. As a consequence thereof, we verified the phenotype of the cells by immunofluorescence staining of α-smooth muscle actin, which is known to be highly expressed in 4-day-old MFB. α-Smooth muscle actin was not detectable in 2-day-old HSC (data not shown).

As we performed a conventional PCR with rat HSC and MFB cDNA, transcripts of all three TGF-β-isoforms were detected on an ethidium bromide gel comparable to the qualitative demonstration of the three isoforms in human MFB by Mangasser-Stephan et al. [21]. Northern blot analysis of TGF-β1 during HSC transdifferentiation to MFB shows a more or less constant signal (Fig. 2A). However, using real-time PCR, in both, quiescent and activated HSC only the TGF-β1 and TGF-β3 mRNAs were expressed in relevant fractions, whereas TGF-β2 mRNA was detectable in low concentrations (Fig. 3A, B). Our results correlate well with in situ hybridization experiments of Milani et al. [6], who could not detect TGF-2-mRNA in normal or fibrotic liver cells. TGF-β2 transcripts were only detected in proliferating human and rat bile duct cells from individuals with liver fibrosis [6]. Likewise, Bissell et al. detected only small amounts of TGF-β2-mRNA in endothelial cells and Kupffer cells using a RNase protection assay [7]. TGF-β2-mRNA was undetectable in hepatocytes and HSC isolated from normal liver [7]. Both, HSC and MFB present all three TGF-β receptors. Particularly with regard to TGF-β receptor type III, which has a high affinity to the TGF-β2-isoform HSC and MFB comply the suppositions for ligand binding. Furthermore, one could speculate that analoguous to human and murine cells *cis*-regulatory elements upstream of the TGF-β2 transcription start site might exist that influence the promoter activity. Additionally, the transcription factor content in HSC and MFB might lead to repression of the TGF-β2 gene as well [22].

TGF-β1 and TGF-β3 mRNA transcripts increased significantly at the second day of HSC culture and one day after trypsinization and reseeding (Fig. 4A). Perhaps the TGF-β1 and TGF-β3 mRNA increase might be a cellular response to stress triggered by the seeding and trypsinization procedure. After this short term increase TGF-β-mRNA concentrations decline to a lower and uniform level. The TGF-β1 and TGF-β3 mRNA transcripts content in HSC and MFB indicate that both isoforms seem to be involved in the transdifferentiation

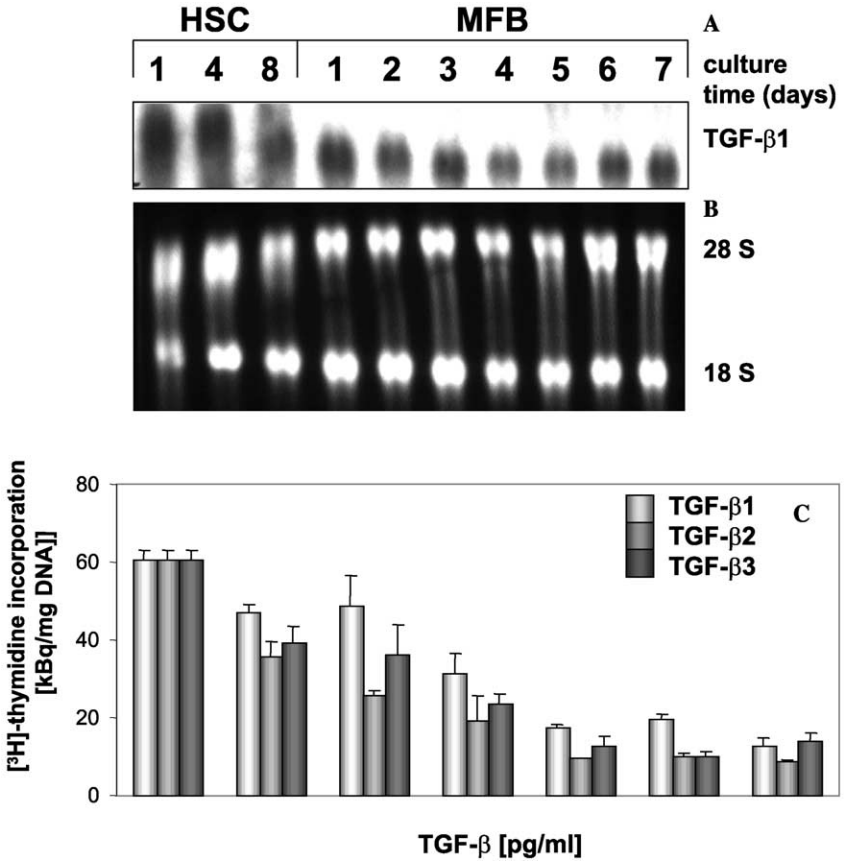

Fig. 2. (A) Northern blot analysis of total RNA (~8 μg per lane) from 1-, 4-, and 8-day-old HSC (lanes 1–3) and 1–7-day-old MFB (lanes 4–10). The blot was hybridized with [^{32}P]-labeled TGF- β1 cDNA [18]. (B) Total RNA purity was verified by a formaldehyde–agarose gel. (C) Effect of TGF-β-isoforms (β1, β2, β3) on HSC proliferation. Incorporated [^{3}H]thymidine was normalized to DNA content. One representative out of two independent experiments.

process and it might also mirror their involvement in profibrogenic process.

Following HSC activation TGF-β1 triggers several gene expressions that modulate proliferation and transdifferentiation of cells. The very low TGF-β2 mRNA expression provokes the question whether this isoform might have antagonistic function to TGF-β1? One feature of TGF-β1 function is the growth inhibition of HSC [23]. Hence, we investigated the antimitogenic influence of each TGF-β isoform on HSC by performing a [^{3}H]thymidine incorporation assay. The addition of TGF-β1, TGF-β2, or TGF-β3 to 2-day-old HSC resulted in a decrease of [^{3}H]thymidine incorporation (Fig. 2c). Growth inhibition was dose-dependent and ranged from 5 to 50 pg/ml TGF-β. This effect was not reinforced by higher concentrations. Our results showed a comparable inhibition of cell proliferation for all three isoforms. Recently, Boumédiene et al. [24] described a bimodal effect of TGF-β-isoforms on proliferation in human chondrosarcoma cells (HCS-2/8), which was dependent on the presence of serum in cell culture and on cell density. They investigated sparse and confluent HCS-2/8 cell cultures either with 10% FCS or serum-free. All three isoforms inhibited cell growth serum independently in sparse cell culture and induced a 2-fold increase of DNA synthesis in serum-fed, confluent cell cultures, which correlate with our experiments using 0.5% FCS. Proliferation of serum-free confluent cultures was stimulated by TGF-β1, inhibited by TGF-β3, and not modulated by TGF-β2 [24]. Qualitative PCR gave clues that the distinct growth response is connected to the TGF-β receptor profile present on these cells [24].

GAPDH mRNA expression during HSC transdifferentiation

To examine wether the GAPDH is an appropriate internal standard in HSC transdifferentiation system we also measured its gene expression by real-time PCR. Absolute mRNA expression for the GAPDH gene expressed during HSC transdifferentiation to MFB was measured to be about 30–90 pg/50 ng total RNA (data not shown). In 3-day-old HSC, GAPDH expression was induced threefold compared with HSC 24 h after seeding. In the course of cultured MFB the GAPDH mRNA

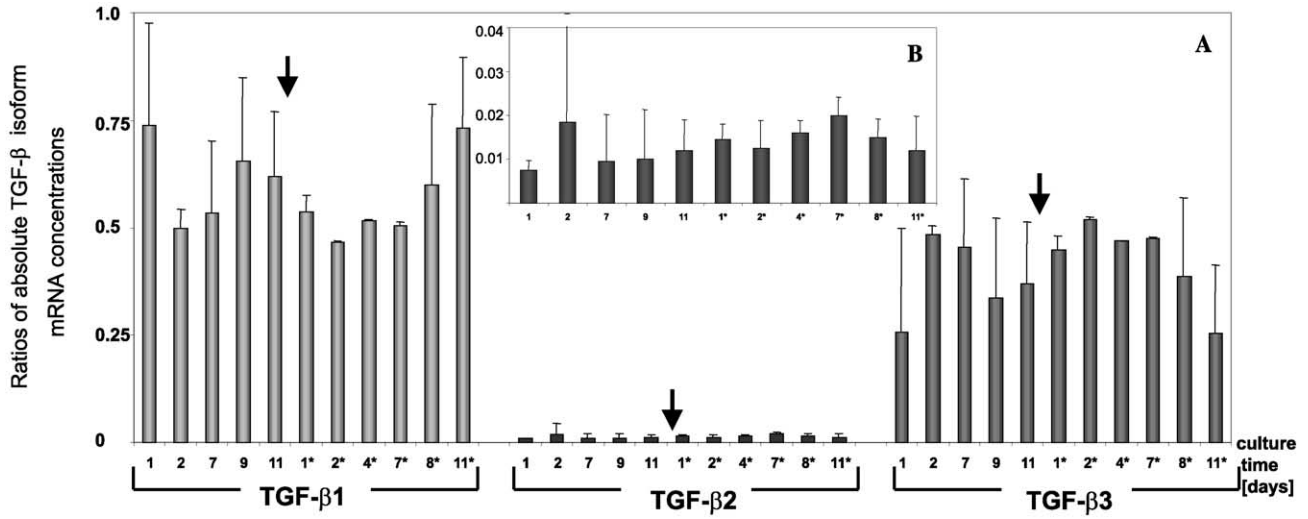

Fig. 3. Comparison of the mRNA expression patterns of the TGF-β-isoforms (β1, β2, β3) during transdifferentiation of rat HSC to MFB. From the same sample absolute mRNA concentrations of all three isoforms were measured and results were added. Each TGF-β-isoform is expressed as a ratio relative to the sum of the absolute mRNA concentrations in this sample. (A) All three isoforms. (B) Expanded ordinate for TGF-β2; [light grey]: TGF-β1, [black]: TGF-β2, [dark grey]: TGF-β3. At the indicated time points total RNA was isolated from primary cultures of HSC (day: 1, 2, 7, 9, 11) and secondary cultures MFB (day: 1*, 2*, 4* 7*, 8*, 11*) and analyzed by quantitative real-time PCR. Arrows indicate trypsinization. Values are the means of three measurements each performed in duplicates from independent experiments.

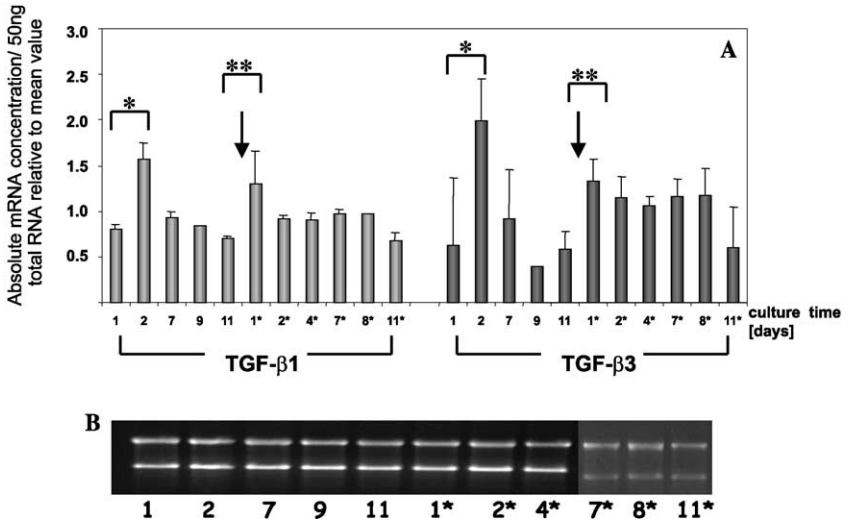

Fig. 4. (A) Quantification of absolute TGF-β1 and TGF-β3 mRNA concentration/50 ng total RNA with a newly developed SYBR Green I-based real-time PCR using LightCycler technique. To compare results of different test series the absolute concentrations of each cell culture series were expressed relative to their respective mean value. At the indicated time points total RNA was isolated from primary cultures of HSC (day: 1, 2, 7, 9, 11) and secondary cultures MFB (day: 1*, 2*, 4* 7*, 8*, 11*) and analyzed by quantitative real-time PCR. Arrows indicate trypsinization. All time points were carried out in three independent experiments, each performed in duplicates. Significance of differences was established by t test (*$p < 0.02$, ** $p < 0.01$); [light grey]: TGF-β1, [dark grey]: TGF-β3. (B) RNA formaldehyde–agarose gel comparing the amount of total RNA of the different samples collected from HSC and MFB rat cell culture.

transcript level changed as well. We suspect that the variations in GAPDH mRNA would not influence Northern blot results, since distinctions of Northern blot results are usually done by using total RNA-samples within the microgram range. However, this house keeping gene is not suitable for a quantitative comparison of mRNA levels using real-time PCR because of its lower detection range. These results were confirmed by other investigators who recently analyzed the regulation of typically used internal standards like GAPDH, β-actin, cyclophilin, 18S RNA, and 28S RNA in their quantification systems [25,26]. Only 18S RNA and 28S

RNA were found to be an appropriate internal standard in their quantification systems. The authors did not note the different PCR efficiencies of investigated gene and internal standard. This fact is heeded by our newly developed gene-specific real-time quantification method. The method described here allows a reproducible and more sensitive quantitative measurement of the TGF-β-isoform mRNAs than conventional methods and, therefore, can be recommended for studies concerned with the role of TGF-β in physiologic processes.

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