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INVESTIGATIVE REPORT

Downregulation of SMAD2, 4 and 6 mRNA and TGFβ Receptor I mRNA in Lesional and Non-lesional Psoriatic Skin

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Transforming growth factor beta (TGFβ) has been suggested to be an effective inhibitor of the increased keratinocyte proliferation in psoriasis. Three TGFβ isoforms are described (TGFβ1, 2 and 3), signalling via a heteromeric receptor complex of TGFβRI and TGFβRII. Receptor binding activates SMAD2, 3 and 4, which translocate into the nucleus and regulate TGFβ-responsive genes. SMAD6 and 7 proteins represent a negative feedback loop inhibiting the TGFβ-SMAD signalling pathway. As TGFβ1 overexpression inhibits keratinocyte proliferation, the aim of this study was to investigate with real-time RT-PCR the expression of TGFβ1, 2 and 3, TGFβRI and TGFβRII and SMAD2, 3, 4, 6 and 7 in lesional and non-lesional psoriatic skin from 13 patients with chronic plaque-type psoriasis as compared to skin from 10 healthy subjects . The study data demonstrate significantly downregulated TGFβRI and SMAD2, 4 and 6 mRNA expression in lesional and non-lesional psoriatic skin. SMAD7 mRNA expression was significantly decreased in lesional psoriatic skin compared with both non-lesional psoriatic skin and healthy skin. A significant TGFβ3 and TGFβRII mRNA upregulation exclusively in non-lesional psoriatic skin but no significant difference in the expression of TGFβ1 and 2 was found. The results of this study suggest that the expression of TGFβ isoforms, receptors and SMADs may be involved in the increased proliferation of keratinocytes in psoriatic skin.

Key words: psoriasis; SMAD; TGFbeta.

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Psoriasis is a chronic immune-mediated inflammatory skin disease. While the cause of psoriasis remains unknown, it appears to result from a combination of genetic and environmental factors. Several studies emphasize the potential role of multiple genes conferring increased susceptibility to psoriasis. Genome-wide scans in the search for psoriasis genes reveal evidence that a major locus of the psoriasis susceptibility gene (PSORS1) is located within the major histocompatibility complex (MHC) on the short arm of chromosome 6 (1). Recent studies highlight the importance of the interleukin (IL)-23 receptor gene as a risk gene not only for psoriasis but also for Crohn's disease (2, 3). Current concepts of psoriasis focus on dendritic cells skewing T cells in the direction of a Th17-differentiation. By production of IL-17 and IL-22 there is direct activation of keratinocytes, which, among other factors, produce antimicrobial peptides, the overexpression of which is a hallmark of psoriasis (4, 5).

Interestingly, the role of negative regulators of proliferation and activation is by far less well understood. Transforming growth factor beta (TGFβ) is suggested to be an effective inhibitor of keratinocyte proliferation in psoriasis (6). TGFβ exists in three isoforms (TGFβ1, TGFβ2 and TGFβ3). The biological function of TGFβ is mediated by specific receptors (TGFβRI, TGFβRII and TGFβRIII). These receptors are transmembrane, heterodimeric complexes with serine/threonine-kinase activities. Binding of TGFβ to its receptor activates the SMAD intracellular signalling pathway (7).

SMAD proteins represent the human homologues of genes first described in *C. elegans* (MAD: mother against decapentaplegic) and in *Drosophila* (SMA: small body size). Eight different SMAD proteins are described in humans and, according to their function, they are classified as "receptor-regulated SMADs" (SMAD1, 2, 3, 5 and 8), "common SMAD" (SMAD4), and "inhibitory SMADs" (SMAD6 and 7). The involvement of SMADs in TGFβ signalling has been studied intensively in wound healing. SMAD3 knock-out mice showed that wound healing is accelerated, while local inflammatory responses are decreased (8). Studies suggest that the beneficial effect of 1α,25-dihydroxyvitamin D_3 (active derivative of vitamin D_3) in psoriasis is mediated at least in part by TGFβ and SMAD signalling (9–11).

To investigate the role of TGFβ and SMAD signalling in psoriasis, biopsies were taken from lesional and non-lesional psoriatic skin, as well as from skin from healthy subjects, and mRNA expression of TGFβ1, 2, 3, TGFβRI and II and SMAD2, 3, 4, 6, and 7 were analysed by real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR).

MATERIALS AND METHODS

Patients

Thirteen patients (8 men, 5 women, mean age 42 years) with chronic plaque-type psoriasis were recruited for the study. None of the patients had received any treatment for at least 4 weeks prior to the investigation. Normal human skin tissue was obtained from 10 healthy subjects who underwent elective surgery for excision of benign naevi. All participants gave their written informed consent prior to participation, and the study followed the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm) and the amendments of the World Medical Association. The study procedure was approved by the local ethics committee.

Tissue samples

In each patient two punch biopsies (5 mm diameter) were taken from lesional and non-lesional skin after local anaesthesia using prilocaine 1%. Biopsies from lesional skin were taken from the margin of plaques. Psoriasis plaques and non-lesional skin were at least 5 cm apart. Control skin samples from healthy subjects were taken with a 5 mm punch biopsy immediately after surgical excision of the naevus from the surrounding skin. All tissue specimens were immediately immersed in RNA-later solution (Qiagen, Hilden, Germany), kept for 24 h at room temperature and finally stored at –20°C until further use.

RNA isolation

Total RNA of the biopsies was isolated using an RNA isolation kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, the biopsy tissue was transferred to 300 µl lysis buffer containing 1% β-mercaptoethanol (Sigma, Deisenhofen, Germany). Thereafter, the tissue was homogenized for 2 sec in iced tubes using an ultrasonic apparatus (Bandelin Sonopuls GM70, Berlin, Germany). After adding 300 µl 70% (v/v) ethanol (Merck, Darmstadt, Germany) total RNA was immobilized on spin columns and treated with DNase before elution in diethylpyrocarbonate-treated (DEPC) water (Fluka, Buchs, Germany). The concentration and purity of RNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer. Ribosomal RNA (28S rRNA and 18S rRNA) was visualized on a 1% agarose gel following electrophoresis to demonstrate its integrity.

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Equal amounts of total RNA from each sample were reverse transcribed into cDNA with standard reagents according to the recommendations of the manufacturer (Gibco-BRL, Karlsruhe, Germany). 1 µg RNA was incubated for 10 min at 70°C with 1 µl oligo dT (0.5 µg/µl) (Gibco-BRL). After incubation RNA was transferred into a reaction mixture containing 4 µl 5 × reverse transcriptase buffer (250 mmol/l Tris-HCl (pH 8.3), 50 mmol/l $MgCl_2$ and 300 mmol/l KCl), 2 µl DTT (100 mmol/l), 1 µl ribonuclease inhibitor (40 U/ml), 1 µl dNTPs (10 mmol/l) and 0.5 µl Superscript II (Gibco-BRL) and incubated at 42°C for 60 min. Transcription was stopped at 95°C for 5 min. Thereafter, the reverse-transcriptase (RT) product was diluted with 80 µl DEPC-water and stored at –20°C until further use.

Real-time quantitative RT-PCR and data analysis was carried out using the LightCycler technique (Roche Diagnostic, Mannheim, Germany). Specific primers are shown in Table I. In these studies, one cDNA preparation that expressed each specific gene of TGFβ, TGFβ receptors or SMADs was arbitrarily employed as the assay standard. The level of expression of each mRNA and their estimated crossing points in each sample was determined relative to the standard preparation using the LightCycler computer software. Total cellular RNA was used as a reference for data normalization (12). Amplification was performed in a total volume of 10 µl including 1 µl RT, 1 µl LightCycler-FastStart Reaction Mix SYBR Green 1 (Roche Molecular Biochemicals), 0.5 µmol/l specific primer, 3 mmol/l $MgCl_2$ with 45 cycles of PCR. Each PCR cycle included denaturation for 1 min at 95°C, annealing of primers at primer specific temperatures (Table I) for 30 sec and elongation at 72°C for 2 min with a final extension at 72°C for 5 min.

To establish the specificity of DNA products, melting curve analysis was carried out after the PCR. For each primer set, agarose gel electrophoresis was also employed to establish the integrity of the PCR reaction and product size.

Statistical analysis

All values were calculated as mean and standard deviation (SD). Statistical significance between groups was analysed by Student's *t*-test. Student's paired *t*-test was used for analysis of the results obtained from lesional and non-lesional psoriatic skin, since we took a paired sample from each patient. We used the unpaired *t*-test for comparison of psoriasis and normal skin. A *p*-value below 0.05 was considered significant.

RESULTS

Expression of TGFβ mRNA

The data obtained from real-time RT-PCR analysis did not reveal different expression of TGFβ1 and TGFβ2 mRNA in lesional or non-lesional psoriatic skin compared with skin from healthy subjects ($p > 0.05$, data not shown). TGFβ3 mRNA expression was significantly upregulated in non-lesional psoriatic skin compared with lesional ($p < 0.01$) and control skin ($p < 0.001$, Fig. 1).

Table I. *Sequence, size of primers and annealing temperature (Tm (°C)) used in real-time RT-PCR assays*

mRNA	Primer sequence (5'-3')	Size (bp)	Tm (°C)
TGFβ1	S-TGG CGA TAC CTC AGC AAC C	405	58.8
	AS-CTC GTG GAT CCA CTT CCA G		
TGFβ2	S-ATC CCG CCC ACT TTC TAC AGA C	565	62.1
	AS-ASCAT CCA AAG CAC GCT TCT TCC		
TGFβ3	S-TAC TAT GCC AAC TTC TGC TC	522	55.3
	AS- AAC TTA CCA TCC CTT TCC TC		
TGFβRI	S- ACG GCG TTA CAG TGT TCT G	358	56.7
	AS- GGT GTG GCA GAT ATA GAC C		
TGFβRII	S- AGC AAC TGC AGC ATC ACC TC	688	59.4
	AS-TGA TGT CTG AGA AGA TGT CC		
SMAD2	S- GGA GCA GAA TAC CGA AGG CA	128	59.4
	AS- CTT GAG CAA CGC ACT GAA GG		
SMAD3	S- AGA AGA CGG GGC AGC TGG AC	511	63.5
	AS-GAC ATC GGA TTC GGG GAT AG		
SMAD4	S- GCA TCG ACA GAG ACA TAC AG	484	57.3
	AS-CAA CAG TAA CAA TAG GGC AG		
SMAD6	S- CAA GCC ACT GGA TCT GTC CGA	321	61.8
	AS- TTG CTG AGC AGG ATG CCG AAG		
SMAD7	S- ATG DTG TGC CTT CCT CCG CT	494	61.4
	AS-CGT CCA CGG CTG CTG CAT AA		

bp: base pairs; TGF: transforming growth factor; RT-PCR: reverse transcriptase-polymerase chain reaction.

Expression of TGFβ receptor mRNA

The TGFβRI mRNA expression was significantly decreased in lesional ($p<0.01$) and non-lesional ($p<0.01$) psoriatic skin compared with control skin. TGFβRII showed a significant increased mRNA expression in non-lesional psoriatic skin compared with lesional psoriatic skin ($p<0.05$) and control skin ($p<0.01$).

Expression of SMAD mRNA

SMAD2 mRNA expression was significantly downregulated in biopsies from lesional and non-lesional psoriatic skin (both $p<0.01$) compared with healthy skin (Fig. 2). Our data did not reveal a significant difference in the expression of SMAD3 mRNA (data not shown). The mRNA expression of SMAD4 was significantly decreased in psoriasis plaques ($p<0.001$) and non-lesional psoriatic skin ($p<0.001$) compared with normal skin (Fig. 2). In lesional psoriatic skin ($p<0.001$) and non-lesional psoriatic skin ($p<0.001$) the mRNA expression of SMAD6 was significantly decreased compared with healthy skin (Fig. 2). The expression of SMAD7 mRNA was significantly downregulated in lesional psoriatic skin compared with healthy skin ($p<0.05$), whereas there was no significant difference in SMAD7 mRNA expression between non-lesional skin and control skin (Fig. 2).

DISCUSSION

TGFβ is suggested to play a central role in various diseases. Mutations in the TGFβ genes, its receptors, and intracellular signalling molecules (SMAD proteins) are associated with the pathogenesis of cancer (13). Despite this important influence of TGFβ on the homeostasis of human tissue there have been only a few studies into the role of TGFβ and its intracellular signalling proteins (SMAD) in the pathogenesis of psoriasis (14–17). As psoriasis is characterized by epidermal hyperproliferation, and several studies showed anti-proliferative effects of TGFβ on keratinocytes (6, 18, 19), we investigated the mRNA expression of TGFβ, its receptors and signalling molecules (SMADs) in psoriatic skin.

Only a few studies describe the expression of TGFβ in psoriatic skin. Kane et al. (20) showed intracellular immunoreactivity of TGFβ1 in basal and suprabasal layers of the epidermis in normal skin, but no staining in lesional psoriatic skin. These results are not in accordance with our findings, since we did not find any differences in TGFβ1 mRNA expression between the analysed skin samples. Since we described mRNA transcript levels of TGFβ1 in whole skin biopsies, it is difficult to evaluate these differing results. In a second study, TGFβ1 was neither detected in normal skin, nor in non-lesional or lesional psoriatic skin,

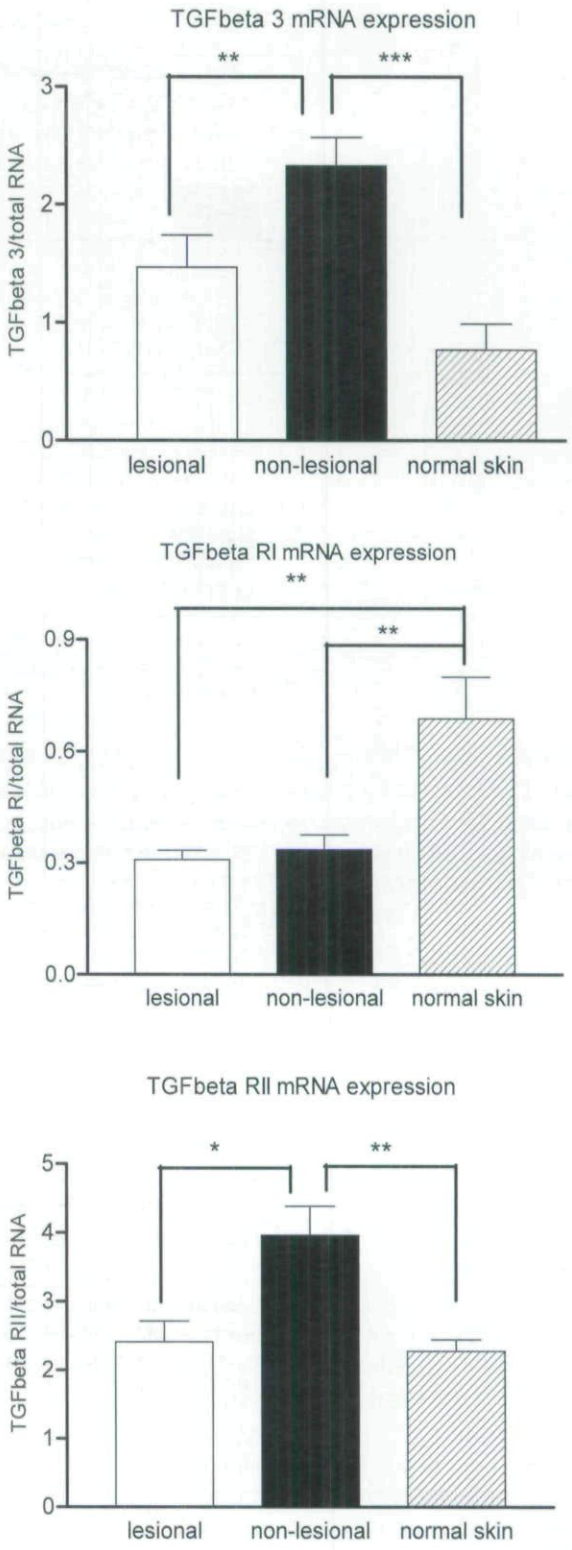

Fig. 1. Transforming growth factor (TGF) β3, TGFβ receptor (TGFβR) I and II mRNA expression analysed by real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) using the LightCycler technique. The data represent mean ± standard deviation. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

whereas TGFβ3 showed subepidermal staining in all skin samples (16). It is difficult to compare our results

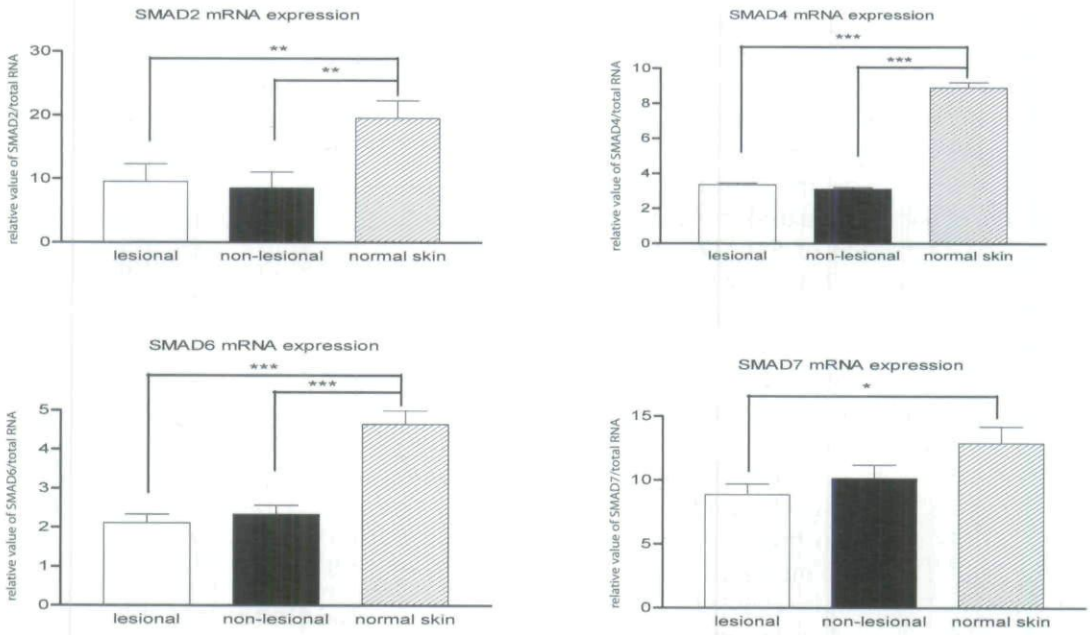

Fig. 2. SMAD2, 4, 6 and 7 mRNA expression analysed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using the LightCycler technique. The data represent mean ± standard deviation. *Significant at $p<0.05$; **significant at $p<0.01$; and ***significant at $p<0.001$.

with the study of Wataya-Kaneda et al. (16), as in the latter TGFβ precursor-specific antibodies were used. Interestingly, irradiation of HaCaT-keratinocytes with narrowband ultraviolet (UV)-B induced upregulation of TGFβ1 in keratinocytes (21) and downregulation in fibroblasts (22). UV-B treatment leads to TGFβ1 induction in epidermal psoriatic skin, which partly explains the beneficial effect in psoriasis. As our data showed upregulation of TGFβ3 mRNA only in non-lesional psoriatic skin, we suggest that increased TGFβ3 levels in non-lesional psoriatic skin might be involved in pathomechanisms responsible for inhibiting epidermal hyperproliferation. TGFβ3 is known as an inhibitory factor in wound healing and is a potential treatment to prevent and reduce scarring (23). The role of TGFβ3 in the development of psoriatic lesions has not been described previously, and studying the effect of TGFβ3 protein on the proliferation of keratinocytes would be highly interesting. As adhesiveness of T-lymphocytes to dermal microvascular endothelial cells can be blocked by TGFβ1, reduction of TGFβ1 expression and function may contribute to lymphocyte infiltration into psoriatic plaques (17). The role of TGFβ1 in psoriasis remains unclear, and further studies investigating TGFβ1 protein expression in psoriatic skin are needed.

The studies of Nockowski et al. (14), Flisiak et al. (15) and Li et al. (18) further support a possible pathogenetic role of TGFβ in psoriasis. Nockowski et al. (14) observed increased serum concentrations of TGFβ1 in patients with psoriasis. Flisiak et al. (15) showed a positive correlation between TGFβ1 plasma levels and the Psoriasis Area and Severity Index (PASI), but their results revealed that TGFβ1 plasma levels do not differ from healthy controls. Flisiak et al. further found a significant correlation between TGFβ1 concentrations in psoriatic scales and disease duration and sedimentation rate (24). These contrasting results of TGFβ1 plasma levels emphasize the importance of new larger studies analysing the protein expression of TGFβ1 in plasma and skin of patients with psoriasis. Li et al. (18) found that transgenic mice overexpressing TGFβ1 in the epidermis develop inflammatory skin lesions with a gross appearance of psoriasis-like plaques and reveal enhanced SMAD signalling in epidermis and dermis (25).

The expression of TGFβ receptors (TGFβR) in psoriasis were investigated by Leivo et al. (26). The authors found intense immunohistochemical staining for both receptors in basal and suprabasal epidermal keratinocytes in normal and non-lesional psoriatic skin, but no staining in the epidermis of lesional psoriatic skin. Our results showed a decreased TGFβRI mRNA expression in lesional and non-lesional psoriatic skin and increased TGFβRII mRNA expression in non-lesional psoriatic skin compared with normal skin. It is difficult to compare these results as Leivo et al. (26) analysed protein expression in the epidermis. However, the results of both studies suggest a possible role of the TGFβ receptors in psoriasis. If TGFβ1 has an antiproliferative effect on the epidermis TGFβ receptors are needed to mediate this effect. Decreased expression of both TGFβ receptors could be a reason for diminished perception of TGFβ1 signalling. This may play an important role in the pathogenesis of psoriasis.

To our knowledge, investigations of SMAD mRNA expression in psoriasis have not yet been performed. The data obtained in our study showed a significant decrease

in the mRNA expression of the inhibitory SMAD6, of the common SMAD4 and of the receptor-regulated SMAD2 in lesional and non-lesional psoriatic skin compared with normal skin. The potential involvement of decreased SMAD6 and 7 expressions in the pathogenesis of psoriasis is substantiated by *in vivo* experiments using narrowband UV-B exposure of normal human skin. After UV-B irradiation, increased expression of SMAD7 could be detected by *in situ* hybridization (27). In the light of these results, it is interesting that our study showed decreased SMAD7 mRNA expression in psoriatic skin compared with normal skin. These data suggest that activation of the TGFβ-SMAD signalling pathway might be involved in the clinical improvement of psoriasis.

The role of SMADs as an intracellular signalling pathway transducing the TGFβ-signal is well established. Binding of TGFβ1 and 2 to the TGFβ receptor complex (TGFβRI and II) leads to increased expression of SMAD2, 4, 6 and 7 (7). Our data revealed decreased expression of SMAD2, 4 and 6 in lesional and non-lesional psoriatic skin compared with normal skin. This points towards decreased TGFβ-signalling. As we found no differences in TGFβ1 and 2 mRNA levels between psoriatic and normal skin, we hypothesize that the decreased expression of TGFβRI is the reason for the decreased SMAD2, 4 and 6 mRNA expression in lesional and non-lesional psoriatic skin.

Further studies are needed to decide whether the TGFβ-SMAD signalling pathway is directly involved in the development of psoriasis or is a side-effect of the immunological processes in psoriatic skin. The interpretation of the results of our study is limited due to the fact that we analysed mRNA transcript levels and we cannot determine what kind of cell might be responsible for changes in transcript levels. Therefore, further studies are needed to describe the protein expression of SMAD and TGFβ in psoriatic skin. The choice of PCR normalization method is crucial in analysing and interpreting PCR results. The PCR data obtained in this study are based on PCR normalization to total RNA. According to Bustin this is the least unreliable method (12), but there still are limitations and risks in interpreting PCR results. An important consideration when using total RNA for normalization is the lack of internal control for RT or PCR inhibitors. All quantitative methods assume that the RNA targets are reversely transcribed and subsequently amplified with similar efficacy. Normalization to total RNA content requires accurate quantification of the RNA sample and, in addition, this method does not provide reliable information about the quality of the RNA, a key consideration when quantitating mRNA levels in fresh tissue. Further problems in analysing PCR data may be associated with methodological errors (cDNA synthesis and PCR analysis). These limitations have been taken into account when interpreting the data obtained in this study.

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