that of TRPV1 (another putative target molecule in mitigating itch) and, furthermore, because certain skin sensitizing, TRPV3-activating agents (e.g., camphor) may also act on TRPV1 (Xu *et al*., 2005), it is proposed that combined approaches targeting both TRPV1 and TRPV3 may prove advantageous in the management of pruritus.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Asakawa M, Yoshioka T, Matsutani T, Hikita I, Suzuki M, Oshima I *et al*. (2006) Association of a mutation in TRPV3 with defective hair growth in rodents. *J Invest Dermatol* 126:2664–72

Chung MK, Lee H, Mizuno A, Suzuki M, Caterina MJ (2004) 2-Aminoethoxydiphenyl borate activates and sensitizes the heat-gated ion channel TRPV3. *J Neurosci* 24:5177–82

Chung MK, Guler AD, Caterina MJ (2005) Biphasic currents evoked by chemical or thermal activation of the heat-gated ion channel, TRPV3. *J Biol Chem* 280:15928–941

Gopinath P, Wan E, Holdcroft A, Facer P, Davis JB, Smith GD *et al*. (2005) Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain. *BMC Womens Health* 5:2

Ikoma A, Steinhoff M, Stander S, Yosipovitch G, Schmelz M (2006) The neurobiology of itch. *Nat Rev Neurosci* 7:535–47

Imura K, Yoshioka T, Hikita I, Tsukahara K, Hirasawa T, Higashino K *et al*. (2007) Influence of TRPV3 mutation on hair growth cycle in mice. *Biochem Biophys Res Commun* 363:479–83

Kochukov MY, McNearney TA, Fu Y, Westlund KN (2006) Thermosensitive TRP ion channels mediate cytosolic calcium response in human synoviocytes. *Am J Physiol Cell Physiol* 291:C424–32

Moqrich A, Hwang SW, Earley TJ, Petrus MJ, Murray AN, Spencer KS *et al*. (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science* 307:1468–72

Nilius B, Owsianik G, Voets T, Peters JA (2007) Transient receptor potential cation channels in disease. *Physiol Rev* 87:165–217

Paus R, Schmelz M, Biro T, Steinhoff M (2006) Frontiers in pruritus research: scratching the brain for more effective itch therapy. *J Clin Invest* 116:1174–86

Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC *et al*. (2002) A heat-sensitive TRP channel expressed in keratinocytes. *Science* 296:2046–9

Purwar R, Werfel T, Wittmann M (2006) IL-13-stimulated human keratinocytes preferentially attract CD4+CCR4+ T cells: possible role in atopic dermatitis. *J Invest Dermatol* 126:1043–51

Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P *et al*. (2002) TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* 418:186–90

Steinhoff M, Bienenstock J, Schmelz M, Maurer M, Wei E, Biro T (2006) Neurophysiological, neuroimmunological, and neuroendocrine basis of pruritus. *J Invest Dermatol* 126:1705–18

Toyoda M, Nakamura M, Makino T, Hino T, Kagoura M, Morohashi M (2002) Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br J Dermatol* 147:71–9

Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D *et al*. (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature* 418:181–6

Xu H, Blair NT, Clapham DE (2005) Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *J Neurosci* 25:8924–37

Yoshioka T, Imura K, Asakawa M, Suzuki M, Oshima I, Hirasawa T *et al*. (2009) Impact of the Gly573Ser substitution in TRPV3 on the development of allergic and pruritic dermatitis in mice. *J Invest Dermatol* 129:714–22

See related article on pg 770

Selection and Validation of Candidate Housekeeping Genes for Studies of Human Keratinocytes—Review and Recommendations

Michael Bär[1], Dorit Bär[2] and Bodo Lehmann[1]

Gene expression analysis using real-time PCR has become an integral part of biomedical research. Appropriate data normalization based on stably expressed housekeeping genes is crucial for reliable results. Thus, candidate housekeeping genes require careful evaluation with regard to the individual experimental system before being selected for studies of human keratinocytes. Future research may be based on published data, as provided by Minner and Poumay in this issue.

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Transcriptional regulation is an important factor in cellular responses. As a consequence, analysis of gene expression profiles has become an integral part of biomedical research, including the field of dermatology. Currently, real-time quantitative reverse transcription PCR (qPCR) is the method of choice for specific and highly sensitive expression profiling of selected groups of target genes. Despite being a powerful technique, qPCR suffers from certain pitfalls, with inappropriate data normalization remaining one of its most important problems. To date, the use of so-called housekeeping genes (HKGs) is the gold standard by which to normalize the mRNA fractions of interest. Usually, these HKGs are among the cellular maintenance genes that regulate basic and ubiquitous cellular functions or code for components of the cytoskeleton (β-actin), major histocompatibility complex (β_2-microglobulin, B2M), glycolytic pathway (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, and phosphoglycerokinase 1, PGK1), metabolic

[1]*Department of Dermatology, Carl Gustav Carus Medical School Dresden, Germany and* [2]*Department of Psychiatry, Carl Gustav Carus Medical School, Dresden University of Technology, Dresden, Germany*

Correspondence: Dr Michael Bär, Carl Gustav Carus Medical School, Department of Dermatology, Fetscherstrasse 74, 01307 Dresden, Germany. E-mail: Michael.Baer@uniklinikum-dresden.de

salvage of nucleotides (hypoxanthine ribosyltransferase, HPRT), protein folding (cyclophilin A, CPA), or synthesis of ribosome subunits (rRNAs), and their expression is presumed to be relatively stable. Thus, an ideal internal control gene should be uniformly expressed during all environmental or experimental conditions in the given experimental system. Coamplification of such HKGs allows the normalization of different amounts and qualities of starting RNA to compensate for varying enzymatic efficiencies in individual samples, because the internal reference gene is exposed to the same preparation steps that the genes of interest are.

Accurate normalization of gene expression data is an absolute prerequisite for reliable results, especially when subtle differences in gene expression are studied. However, no definite or universal HKGs with proven invariable expression between cells or experimental conditions has as yet been identified. There is increasing evidence that many commonly used HKGs are not as resistant to internal and external environmental changes as previously thought. Their individual expression may vary as a result of coamplification of pseudogenes, neoplastic growth, or experimental treatment and thus may seriously compromise the correct interpretation of the corresponding results (Goidin *et al.*, 2001). Until now, the most commonly used reference genes included β-actin, GAPDH, HPRT, and 18s-rRNA. Indeed, we must keep in mind that these HKGs are merely historical holdovers and that they were reliably accepted as adequate references for many years in non- or semiquantitative techniques such as northern blot, RNase protection assays, and conventional RT-PCR. Although they are acceptable for these semiquantitative techniques, their consecutive qualification as appropriate HKGs for the highly sensitive qPCR has recently been re-evaluated, resulting in strategies to control for their instability, such as using sets of control genes and calculating normalization factors with statistical algorithms. For this purpose, Vandesompele *et al.* (2002) worked out an applet called geNorm (available at http://medgen.ugent.be/~jvdesomp/genorm/) that enables the consecutive elimination of less stable HKGs among a selection of putative HKGs and re-evaluation of the remaining genes, finally resulting in a selection of a suitable HKG for an individual system. For accurate subsequent averaging of the control genes, the authors proposed using the geometric mean of HKG expression as a normalization factor instead of the arithmetic mean, because it helps to control for possible outlying values and abundance differences among the different genes. Alternatively, Bestkeeper (available at http://www.gene-quantification.de/bestkeeper.html) or Normfinder (http://www.mdl.dk/publicationsnormfinder.htm) may be options for validating HKGs.

> Normalizing for gene expression is no longer simple.

In many studies, normalization is based on coamplification of a single HKG. According to Vandesompele and co-workers (2002), the best option is to measure a panel of multiple HKGs. However, this method usually requires extensive and expensive practical validation to identify a combination of reference genes appropriate for each individual experimental condition.

De Kok *et al.* (2005) identified HPRT as the best single and widely stable reference gene of 13 frequently used HKGs with independent functions in cellular maintenance (e.g., β-actin, B2M, GAPDH, CPA, HPRT, porphobilinogen deaminase, and PGK1) using qPCR in 80 normal and tumor samples of different tissues, including skin. The authors even suggest HPRT as an accurate and economic alternative for the measurement of multiple HKGs (as postulated by Vandesompele *et al.*, 2002) for future studies in cancer research and tumor diagnostics until a definite gold standard has been identified. Moreover, according to de Kok and co-workers (2005), most research applications would not require more than one HKG, because most of the suitable HKG combinations identified by Vandesompele *et al.* (2002) already included HPRT. However, in our opinion, an apparently widely stable HKG may be inappropriate if the given experimental system influences only HPRT. On the other hand, a meta-analysis of more than 13,000 human gene array samples resulted in the identification of novel candidate HKGs with enhanced stability among a multitude of different cell types and varying experimental conditions, with none of the commonly used HKGs found in the top 50 stably expressed genes (de Jonge *et al.*, 2007).

If the coamplification of a panel of HKGs is preferred, HKGs with independent functions in cellular maintenance should be chosen, because selecting genes that share identical biochemical pathways could bias analyses. Additionally, one must be aware that HPRT is relatively lowly expressed and thus applicable only for the normalization of lowly expressed target genes. For highly expressed genes of interest, alternative HKGs with comparable expression levels should be chosen. For skin tissues, β-actin has been described as a high-expression HKG. However, expression of β-actin and GAPDH varies across tissues, among cell types, and during cell proliferation and development. Additionally, the GAPDH-mRNA level is widely altered in cultured cells in response to various stimuli, including hypoxia, insulin, dexamethasone, mitogens, and EGF (Zhong *et al.*, 1999). There have been numerous reports concerning GAPDH regulation in cancer, tumor samples, and cultured cells, as well as GAPDH responses to experimental treatment (Oliveria *et al.*, 1999; Schmidt *et al.*, 2006).

The literature on HKGs in keratinocytes contains an abundance of inconsistent findings. Particularly in human epidermal keratinocytes, whose phenotype depends on a complex differentiation program and on culture conditions, special attention must be paid to the choice of HKG. The recent findings of Minner and Poumay (2009, this issue) support the choice of TATA box–binding protein (TBP) or the ribosomal proteins large 13A or P0, when studying differentiation of keratinocytes. Because UV radiation is the most important carcinogenic environmental stressor of human

skin, UV-induced changes in keratinocytes are also widely studied. UV radiation damages DNA directly and via the production of reactive oxygen species, interfering with basic cellular metabolic processes. Therefore, selection of HKGs in studies including UV irradiation is of particular importance.

Balogh *et al*. (2008) recently analyzed the mRNA expression of 10 HKGs (e.g., 18s-rRNA, GAPDH, β-actin, B2M) in neonatal human epidermal keratinocytes after UVB exposure. Based on geNorm and Normfinder analysis, succinate dehydrogenase complex subunit A (SDHA) was found to be the best individual reference gene and SDHA and PGK1 comprised the most suitable combination. Interestingly, GAPDH, regardless of its low stability, showed no apparent UVB-related regulation. However, using *in situ* hybridization, Wu and Rees (2000) demonstrated that UVB exposure would result in highly significant increases in GAPDH expression throughout the epidermis.

Particular attention must be paid to HKG in diseased skin, and few data based on qPCR have been published. Using *in situ* hybridization, however, Wu and Rees (2000) revealed an increased expression of GAPDH in all areas of the epidermis of psoriatic plaque compared with normal skin. Interestingly, using qPCR, Allen *et al*. (2008) found important differences in suitable HKGs for different types of keratinocytes (e.g., HaCaT and adult vs. neonatal keratinocytes). Any combination of CPA, β-glucuronidase, TBP, B2M, ubiquitin C, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (zeta polypeptide), HPRT, and large ribosomal protein P0 was found to be appropriate as an HKG when using HaCaT or adult keratinocytes, whereas only the expression of TBP, B2M, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (zeta polypeptide), and β-glucuronidase was sufficiently stable for the normalization of expression data in juvenile keratinocytes. These findings indicate the impact of using keratinocytes of different origin. Finally, when using tissue samples consisting of different (i.e., contaminating) cell types, the HKG should be stably expressed in all the included cells to avoid rarefaction effects.

Taking all this into consideration, we present the following recommendations. Based on a proper validation process, investigators should be able to demonstrate that the reference gene of choice is suitable for the individual experimental system. Economic considerations may require basing the study on published HKG data. Thus, the corresponding HKG may be chosen from the validated preselection of putative HKGs, as provided by Minner and Poumay (2009), Allen *et al*. (2008), or Balogh *et al*. (2008). To obtain the most reliable results, more than one HKG should be used. For practical purposes, we recommend the initial selection of two HKGs with independent functions in cellular maintenance (see above) for each experimental condition. The ratio of the expression of these two genes should be relatively constant. According to Huggett *et al*. (2005), an acceptable level of variability will depend on the degree of resolution required for the experiment. On one hand, even if the chosen gene is variable, it may not matter as long as the intergroup difference of the target gene being measured is greater than the corresponding reference gene variation. For example, a reference gene revealing a variation of 1 log may not be ideal, but nevertheless sufficient to measure a 2-log change in the gene of interest. On the other hand, the smaller the variation in the target gene, the more stable the selected HKG should be. If the ratio of the two selected HKGs varies, one or both of these genes is not constantly expressed, and one or more HKGs must be analyzed to identify the most suitable pair of HKGs.

In contrast, Vandesompele *et al*. (2002) recommend using at least three of the most stable internal control genes for geNorm-based calculation of a qPCR normalization factor and stepwise inclusion of more control genes until the next added gene does not significantly alter the newly calculated normalization factor. However, this approach may be disproportionate when only a few target genes are studied or only minimal amounts of RNA are available. Finally, not only the selection of suitable HKG but also the elimination of further causes of inconsistent results of qPCR are important in providing an adequate primer design with the highest specificity.

Minner and Poumay highlight the importance of a careful evaluation of candidate HKGs when studying human epidermal keratinocytes, providing a helpful and carefully validated preselection of useful HKGs, especially for studies of differentiation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Allen D, Winters E, Kenna PF *et al*. (2008) Reference gene selection for real-time rtPCR in human epidermal keratinocytes. *J Dermatol Sci* 49:217–25

Balogh A, Paragh G Jr, Juhasz A *et al*. (2008) Reference genes for quantitative real time PCR in UVB irradiated keratinocytes. *J Photochem Photobiol B* 93:133–9

de Jonge HJ, Fehrmann RS, de Bont ES *et al*. (2007) Evidence based selection of housekeeping genes. *PLoS ONE* 2:e898

de Kok JB, Roelofs RW, Giesendorf BA *et al*. (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest* 85:154–9

Goidin D, Mamessier A, Staquet MJ *et al*. (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal Biochem* 295:17–21

Huggett J, Dheda K, Bustin S *et al*. (2005) Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 6:279–84

Minner F, Poumay Y (2009) Candidate housekeeping genes require evaluation before their selection for studies of human epidermal keratinocytes. *J Invest Dermatol* 129:770–3

Oliveira JG, Prados RZ, Guedes AC *et al*. (1999) The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase is inappropriate as internal control in comparative studies between skin tissue and cultured skin fibroblasts using Northern blot analysis. *Arch Dermatol Res* 291:659–61

Schmidt U, Fuessel S, Koch R *et al*. (2006) Quantitative multi-gene expression profiling of primary prostate cancer. *Prostate* 66:1521–34

Vandesompele J, De Preter K, Pattyn F *et al*. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034

Wu YY, Rees JL (2000) Variation in epidermal housekeeping gene expression in different pathological states. *Acta Derm Venereol* 80:2–3

Zhong H, Simons JW (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res Commun* 259:523–6