Physiol Genomics 43: 604–610, 2011.
First published December 21, 2010; doi:10.1152/physiolgenomics.00131.2010.

CALL FOR PAPERS | *MicroRNA and Tissue Injury*

Normalization strategy is critical for the outcome of miRNA expression analyses in the rat heart

Trond Brattelid,* Eva-Katrine Aarnes,* Erik Helgeland, Steinar Guvåg, Heike Eichele, and Anne K. Jonassen

Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen, Norway

Submitted 7 July 2010; accepted in final form 20 December 2010

Brattelid T, Aarnes EK, Helgeland E, Guvåg S, Eichele H, Jonassen AK. Normalization strategy is critical for the outcome of miRNA expression analyses in the rat heart. *Physiol Genomics* 43: 604–610, 2011. First published December 21, 2010; doi:10.1152/physiolgenomics.00131.2010.— Since normalization strategies plays a pivotal role for obtaining reliable results when performing quantitative PCR (qPCR) analyses, this study investigated several miRNA normalization candidates in regards to their efficiency as normalization standards in the ischemic reperfused ex vivo rat heart, with special reference to regulation of the miRNAs miR-1 and miR-101b. The possibility of including primers for several miRNAs in one reverse transcription (RT) reaction was also investigated. Langendorff perfused rat hearts were subjected to 30 min regional ischemia and 0, 1, 5, 15, or 120 min reperfusion. Total RNA was isolated and reverse transcribed for miRNA qPCR analysis. Normalization candidates were evaluated by the NormFinder and geNorm algorithms and the following stability expression rank order was obtained: sno202 < U6B < U87 < snoRNA < 4.5S RNA A < Y1 < 4.5S RNA B < *GAPDH*. Applying U6B as a normalizer it was found that miR-1 and miR-101b was downregulated in the ischemic reperfused myocardium. Furthermore, up to three primers could be included in one RT reaction by replacing RNase-free water with two supplemental sets of primers in the TaqMan MicroRNA assay protocol. This study demonstrates the importance of validating normalization standards when performing miRNA expression analyses by qPCR, and that miR-1 and miR-101b may play an important role during early reperfusion of the ischemic rat heart.

ischemia; reperfusion; microRNA regulation

microRNAs (miRNAs or miR) are small, noncoding RNAs (ncRNA; 17–25 nucleotides) that posttranscriptionally regulate the expression of genes, affecting several biological processes such as development, differentiation, apoptosis, and oncogenesis (7, 22, 29, 30). They also play an important role in the pathophysiology of the heart, e.g., in cardiac hypertrophy (8, 24) and acute myocardial infarction (AMI) where expression of several miRNAs such as miR-1 and miR-101b are significantly regulated hours to days after an ischemic insult (13, 25, 27). Quantitative PCR (qPCR) is a powerful technique ideal to evaluate the expression profile of miRNAs. For reliable comparison of miRNA expression levels in qPCR, normalization is a necessity to correct for intra- and intergroup variations in between samples and runs. The criteria for a "perfect" mRNA normalization standard in qPCR expression profiling also applies to miRNAs: *1*) equal transcription level in all tissues and cell types at all stages of development and *2*) stable transcription levels during external or internal stimulation (23). No normalization standard has yet proven to be ideal, and it is therefore crucial to verify the expressional stability of putative normalization standard candidates in each experimental setup to get reliable interpretation of results. At present no such validation of normalization standards for miRNA rat heart expression studies has been performed.

To increase normalization accuracy and decrease experimental variation in qPCR experiments, an internal standard subjected to identical treatments as the target of interest should be included to serve as a control for all steps required to obtain the final PCR product (15). Currently, commercial available kits like the TaqMan MicroRNA assay (Applied Biosystems) for expression profile screening by qPCR is limited to one ncRNA in each reverse transcription (RT) reaction. This approach lacks the ability to correct for ncRNA expression level in the same RT reaction. Although the RT reaction is supposed to be relatively stable, it is important to normalize the ncRNA of interest to one or several normalization standards reverse transcribed in the same reaction to account for variation in RT efficiency between samples and runs.

Here we report a strategy to evaluate ncRNA(s) of interest including normalization standard(s) in the same RT reaction. This study also identifies several reliable normalization standards when investigating miRNA expression in the ex vivo Langendorff perfused rat heart exposed to a lethal ischemic insult and reperfusion. Furthermore, the consequences of normalizing the expression level of miR-1 and miR-101b applying one of the more stable (U6B) versus the least stable (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*) candidate was investigated.

MATERIALS AND METHODS

Langendorff Perfusion Procedure

All experiments were approved by Norwegian Laboratory Animals Authority and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Isolation of total RNA and reverse transcription qPCR (RT-qPCR) experiments were performed in accordance with the MIQE guidelines (6).

Hearts from male Wistar rats (270–350 g, $n = 40$) anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) with 200 IU heparin were excised and placed in ice-cold Krebs-Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 11 mM D-glucose, 4.7 mM KCl, 1.22 mM $MgSO_4 \times 7\ H_2O$, 1.21 mM KH_2PO_4, 2.4 mM $CaCl_2 \times 2$

* T. Brattelid and E.-K. Aarnes contributed equally to this work.

Address for reprint requests and other correspondence: E.-K. Aarnes, Dept. of Biomedicine, Jonas Lies vei 91, 5009 Bergen, Norway (e-mail: e-k.aarnes@live.no).

H_2O) followed by retrograde aortic perfusion in the Langendorff setup. The hearts were stabilized for 20 min before being subjected to 30 min regional ischemia and 120 min of reperfusion as previously described (16). In short, a 3-0 silk suture was passed around the main branch of the left coronary artery with the ends threaded through a small vinyl tube to form a snare. Regional ischemia was achieved by pulling and reperfusion by releasing the snare. Heart tissue to be analyzed for miRNA regulation and expressional stability of miRNA normalization standards using RT-qPCR were collected at 0, 1, 5, 15, and 120 min ischemic reperfusion (Fig. 1). At the end of each experiment the silk suture was securely tightened before 0.2% (wt/vol) Evans Blue (Duke Scientific, Palo Alto, CA) was infused to demarcate the risk zone from the nonrisk area. Based on this staining, the regions were separated and isolated heart tissue was stored in RNAsafer (Omega Bio-Tek, Norcross, GA) at 4°C overnight and subsequently frozen at −20°C until further processing.

Isolation of Total RNA From Rat Heart

To isolate total RNA, myocardial tissue samples (50–100 mg) were homogenized in TRIzol (Invitrogen, Carlsbad, CA), DNase I treated (Takara, Shiga, Japan) and precipitated with 2.5 volumes EtOH (96%), 0.1 volumes NH_4Ac (5 M; Ambion, Austin, TX), and 1 μl GlycoBlue (Ambion). Finally, total RNA was purified using RNeasy columns (RNeasy Mini Kit Cleanup Kit; Qiagen, Hilden, Germany).

Total RNA purity and concentration was determined by measuring absorbance in the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA), and RNA samples with optical density (OD) 260/280 and OD 260/230 ratios of 1.8–2.0 were included. Furthermore, to ensure appropriate quality of the total RNA isolation procedure the RNA integrity was assessed by the Agilent 2100 Bioanalyser.

RT-qPCR

Priming test of small ncRNAs. Small ncRNAs were reverse transcribed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), which includes hairpin stem loop primers for only one specific ncRNA. Kits with primers for miR-1, miR-101b, U6B, sno202, 4.5S RNA A, 4.5S RNA B, snoRNA, Y1, and U87 were used. To test the possibility of including more than one RT primer per RT reaction, the number of primers and/or total reaction volumes in the manufacturer protocol was modified. To ensure equal amount of template, a fixed ratio of 30 ng total RNA per 15 μl reaction volume was used. This priming study contained five groups: *group A*, one primer in 15 μl reaction volume (standard protocol); *group B*, three primers in 15 μl reaction volume; *group C*, three primers in 30 μl reaction volume; *group D*, six primers in 30 μl reaction volume; and *group E*, nine primers in 45 μl reaction volume. Each 15 μl RT reaction volume included 5 μl 10× buffer, 2 μl DNase I, and 1 μl ribonuclease inhibitor and were incubated at 16°C for 30 min, 42°C for 30 min, and then at 85°C for 5 min in a PTC-200 Peltier Thermal Cycle PCR machine (MJ Research, Waltham, MA).

ncRNA normalization standard test. Based on the results obtained in the priming test described above, RT reactions in the following evaluation of ncRNA normalization standards included three primers in 15 μl reaction volume (*group B*), resulting in three experimental RT groups: *group 1* contained primers for U6B, sno202 and 4.5S RNA A; *group 2* contained primers for U6B, 4.5S RNA B and snoRNA; *group 3* contained primers for U6B, Y1 and U87. Even though RT assays are relatively stable, this was evaluated by including U6B in all three groups to confirm that this also applies when using three RT primers per reaction. Finally, to examine the effect of normalization on miRNA expression analyses a fourth independent group was run: *group 4* contained primers for U6B, miR-1, and miR-101b.

The miRNA qPCR analysis was evaluated in triplicate with 3 μl of template cDNA (diluted 1:3), 10.5 μl TaqMan 2× Universal PCR Mastermix, 1 μl primer, and RNase-free water to a total volume of 21 μl per reaction. Samples (20 μl each) were loaded on 384-well plates and run in an ABI Prism 7900 qPCR machine (Applied Biosystems; 10 min at 95°C following 40 cycles of 95°C for 15 s and 60°C for 60 s).

RT-qPCR analysis of GAPDH messenger RNA. For cDNA synthesis we incubated 2.5 μg of total RNA with 1.2 μl oligo dT (25 mM, Thermo Scientific) and 13 μl of RNase-free water at 65°C for 5 min, then cooled it to room temperature, before adding 2 μl DTT (Stratagene, La Jolla, CA), 2 μl 10× AffinityScript RT-buffer (Stratagene), 0.8 μl dNTP mix (100 mM, Invitrogen), and 1 μl AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) to a total volume of 20 μl. Samples were reverse-transcribed at 50°C for 60 min, 70°C for 15 min, and then at 4°C when completed in a PTC-200 Peltier Thermal Cycle PCR machine (MJ Research).

The mRNA qPCR analysis was evaluated in triplicate with 6 μl template cDNA (diluted 1:5), 0.7 μl forward primer (10 μM), 0.7 μl reverse primer (10 μM), and 11 μl 2× Mastermix (Messagreen, Eurogenetech; for SYBR Green assay) or qPCR Mastermix Plus (Eurogenetech, for hydrolysis probe assay) in a total volume of 22 μl per reaction. For hydrolysis probe assay 0.9 μl water were replaced with the same volume probe (5 μM). All samples were run with identical running conditions as described for ncRNAs. Subsequently, melting curve analysis was performed at 60–95°C (gradually) for 10 min to confirm primer specificity for the SYBR Green assay. Primers (Invitrogen) and probe (Eurogentech) sequences for *GAPDH* are listed in Brattelid et al. (3). The *GAPDH* hydrolysis probe was labeled with 5′-FAM and quenched with 3′-black hole quencher.

Statistical Analysis

Amplification plots were generated for each sample during qPCR using Sequence Detector Software (SDS version 2.2, Applied Biosystems), which automatically calculated a quantification cycle (C_q) value from each amplicon. Relative initial RNA amount of each RNA was calculated (5) and imported into geNorm (28) and NormFinder (1) for assessment of expressional stability of normalization candidates. Results were analyzed by the nonparametric Mann-Whitney test due to non-Gaussian distribution, and $P < 0.05$ was considered statistically significant. Data are expressed as mean ± SE.

RESULTS

Test of Priming Method

The TaqMan MicroRNA assay is designed for RT of only one small ncRNA per reaction. In this study we explored the possibility of reverse transcribing several ncRNAs by increasing the number of RT primers (1–9 primers) and the total RT reaction volume (15–45 μl) in one RT reaction, allowing

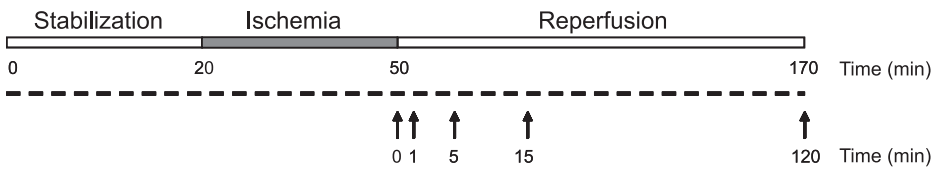

Fig. 1. Experimental protocol. All hearts were subjected to 20 min stabilization before 30 min regional ischemia followed by reperfusion. Dotted lines indicate infusion of Krebs-Henseleit buffer; arrows indicate time points for tissue collection (0, 1, 5, 15, and 120 min of reperfusion); $n \geq 4$ in each group.

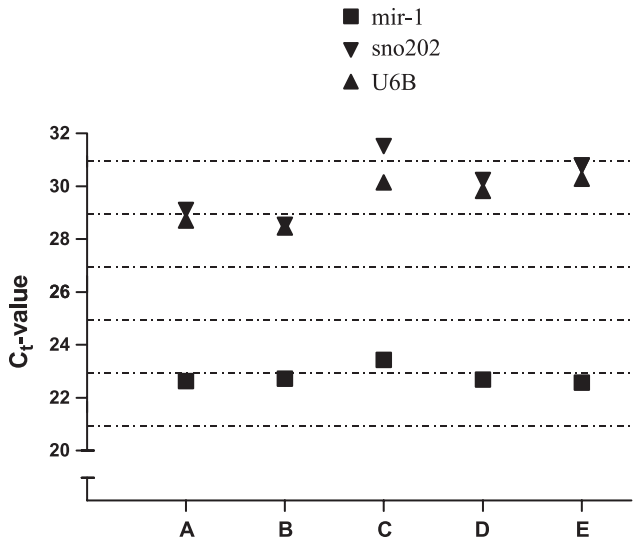

Fig. 2. Effect of increasing number of reverse transcription (RT) primers and total volumes in 1 RT reaction. To evaluate effects of increasing number of RT primers and total reaction volume in 1 RT reaction triplicate quantification cycle (C_q) scatter variation in 3 samples were examined. Variation should be <0.5 C_q when comparing standard and modified protocol in identical samples. Sample reverse transcribed with 1 primer per 15 μl reaction (standard protocol, *column A*), 3 primers per 15 μl reaction (*column B*), 3 primers per 30 μl reaction (*column C*), 6 primers per 30 μl reaction (*column D*), and 9 primers per 45 μl reaction (*column E*). Representative results are presented for miR-1, sno202, and U6B as means of triplicate ± SE.

identical RT conditions for the ncRNA(s) of interest and the normalization candidate(s).

Initially, the expression level of miR-1, sno202, and U6B were analyzed in three different samples in independent RT reactions (data not shown). The samples were reverse transcribed with one primer (miR-1, sno202, or U6B) per 15 μl reaction volume according to the manufacturer's assay protocol. When applying the two small ncRNAs sno202 and U6B, both suggested to be suitable normalization standards for miRNA expression analysis, and the muscle specific miR-1, all triplicates were expressed with a sample C_q scatter variation of $\leq$ 0.3. It is recommended that qPCR samples should not exceed a variation in between reaction replicates of >0.5 C_q (19). Hence, a comparison of replicates from modified and standard protocols of identical samples would give an indication of the applicability of modifications. If unaffected, C_q values resulting from modifications should merge and vary <0.5 C_q in identical samples from values obtained from the standard protocol supplied by the manufacturer. The relationship between C_q value variability obtained using three ncRNA RT primers in 15 μl total volume (Fig. 2, *column B*) corresponded well with the standard one-primer protocol (Fig. 2, *column A*) with a C_q variability <0.5 for all ncRNAs. Further increase in volume (30–45 μl) and number of ncRNA RT primers (3–9 primers) in the RT reaction increased variability between C_q values for sno202 and U6B compared with standard protocol (Fig. 2, *columns C–E*) with $\geq$1 C_q. The variability of miR-1 in contrast was only affected by >0.5 C_q when the ratio of total reaction volume to number of primers was increased (three primers in 30 μl reaction volume) (Fig. 2, *column C*), indicating that highly expressed ncRNAs such as miR-1 are less affected by modifications than other less-expressed ncRNAs such as U6B and sno202 (reflected by C_q values in Fig. 2). This implies that it is possible to include up to three ncRNA primers in one standard RT reaction with tolerable effect on C_q values.

Determination of the Most Stable miRNA Normalization Candidates in the Ex Vivo Rat Heart

Several small ncRNAs are available as normalization standards for qPCR miRNA expression analyses in rat tissue, namely small cytoplasmic RNAs (scRNA), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) such as Y1, U6B, 4.5S RNA A, 4.5S RNA B, snoRNA, sno202, and U87. In addition, messenger RNAs (mRNAs) such as *GAPDH* are used as reference genes (11, 17, 21). The relative initial amount of RNAs from five experimental groups of ex vivo Langendorff perfused rat hearts (0, 1, 5, 15, and 120 min of reperfusion; Fig. 1) were analyzed in NormFinder and geNorm to test expressional stability of the eight above mentioned normalization candidates. In both programs, stable expression corresponds with low values. NormFinder and geNorm analyzes of the reference RNA expression demonstrated a very high correlation between the stability value generated by the respective algorithms ($R^2 = 0.996$) (Fig. 3). *GAPDH* proved to be the least stable normalization candidate in the isolated ischemic-reperfused rat heart irrespective of qPCR assay used, hydrolysis probe or SYBR Green, and showed the highest variability according to values obtained in NormFinder and geNorm (Fig. 3). The overall stability rank order of the ncRNA normalization standards investigated was sno202 $<$ U6B $<$ U87 $<$ snoRNA $<$ 4.5S RNA A $<$ Y1 $<$ 4.5S RNA B $<$ *GAPDH* (Fig. 3).

Based on the finding that it is possible to include up to three ncRNA primers in one standard RT reaction with minute

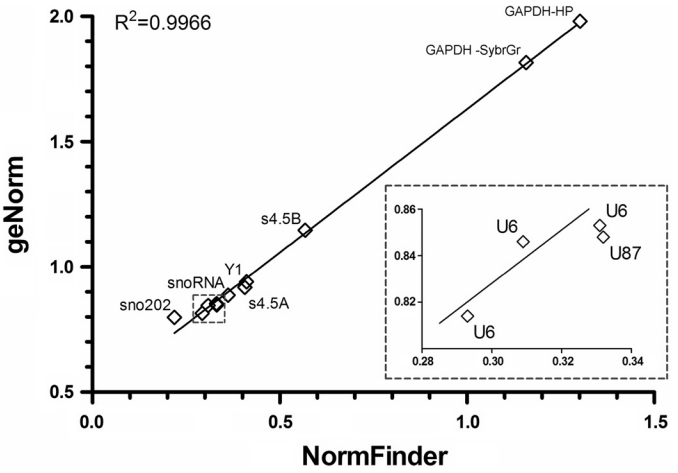

Fig. 3. NormFinder and geNorm analyses of normalization standard candidates. For each normalization standard candidate, geNorm M values and NormFinder stability values were calculated. Values correspond to expressional stability, with low values representing most stable candidates. The geNorm M value and NormFinder stability value demonstrated good correlates for the eight candidates examined in ex vivo Langendorff perfused rat hearts. U6B was included in all ncRNA RT reactions to evaluate stability of the RT assays, and the 3 independent values obtained in NormFinder and geNorm correlated well (*inset*). Independent of whether the qPCR assay was performed with either SYBR Green (*GAPDH*-SybrGr) or hydrolysis probe (*GAPDH*-HP), the stability of *GAPDH* as normalization standard gene was almost identical. All ncRNA RT reactions included 3 primers in a 15 μl reaction volume according to the following groups: *1*) U6B, sno202, and 4.5S RNA A; *2*) U6B, 4.5S RNA B, and snoRNA, *3*) U6B, Y1, and U87.

effects on C_q values (Fig. 2), the ncRNA normalization candidate U6B was included in all ncRNA RT reactions to test the stability of the RT assay (Fig. 3, inset). U6B showed an almost identical ranking value independent of RT reaction (0.29–0.33 in NormFinder and 0.81–0.85 in geNorm) confirming that the ncRNA RT reaction is both stable and reproducible.

Impact of Normalization on miRNA Expression Profiles of miR-1 and miR-101b

To demonstrate the importance of applying the correct miRNA normalization strategy, we evaluated miRNAs expected to be regulated during ischemia-reperfusion. Several studies have shown that this applies to miR-1, and therefore this miRNA was an obvious candidate (13, 25). We also evaluated miR-101b, shown to be regulated in ischemia-reperfusion by preliminary studies in our group and during cardiac ischemia (27). The expression of miR-1 and miR-101b were compared when normalized to the least stable normalization candidate (*GAPDH*) versus a more stable normalization candidate (U6B) (Fig. 4).

The expression of miR-1 is not affected by the normalization standard chosen (Fig. 4, *A* and *B*). When normalized to either *GAPDH* or U6B, heart tissue samples showed a significant and sustained reduction of miR-1 expression from 1 min of reperfusion compared with the ischemic group (0 min) (Fig. 4, *A* and *B*). When heart tissue was analyzed for miR-101b expression the choice of normalization candidate did have a major impact on the obtained results. No statistical differences were observed between the groups when miR-101b was normalized to *GAPDH* (Fig. 4*C*). In contrast, if miR-101b was normalized to U6B there was a significant reduction in expression at 15 and 120 min of reperfusion compared with the ischemic group (0 min) (Fig. 4*D*). The suitability of U6B over *GAPDH* as normalization standard for analyzing expression of miRNA is also emphasized by the higher coefficients of variance value obtained when miR-1 and miR-101b is normalized to *GAPDH*,

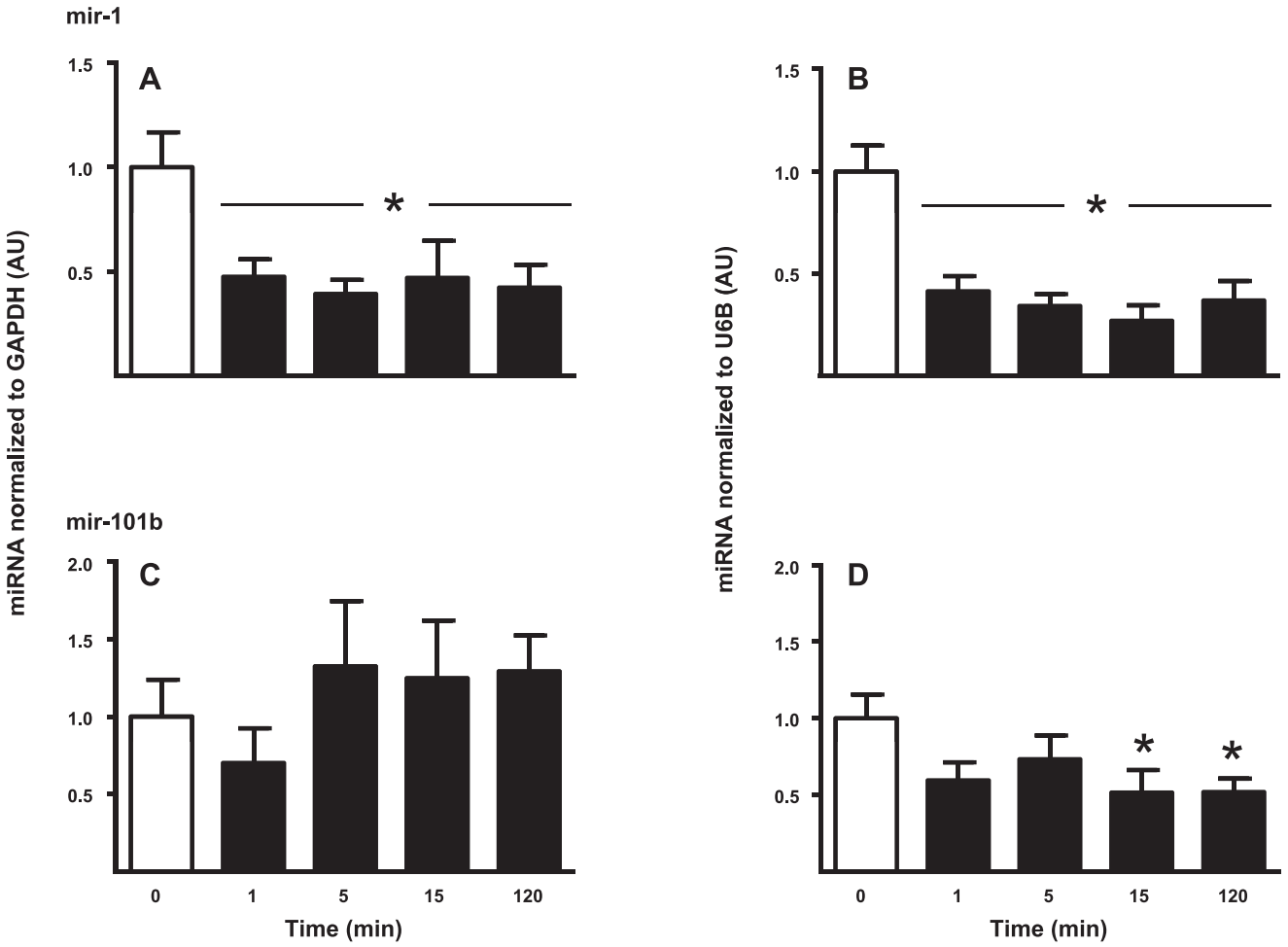

Fig. 4. Relative expression of miRNAs when normalized to *GAPDH* or U6B in reperfused ischemic rat myocardium. All hearts were subjected to 20 min of stabilization prior to induction of regional ischemia (30 min) and reperfusion. Heart tissue for miRNA analysis was collected at 0, 1, 5, 15, and 120 min (see experimental protocol, Fig. 1). *A*: expression of miR-1 was significantly reduced already at 1 min of reperfusion and sustained throughout the reperfusion period when normalized to *GAPDH*. *B*: the same trend in regulation was observed using U6B as normalization standard. *C*: when miR-101b was normalized to *GAPDH*, no significant regulation occurred in the reperfused ischemic myocardium. *D*: in contrast, expression level of miR-101b was significantly decreased at 15 and 120 min of reperfusion compared with ischemic group (0 min) when normalized to U6B. The discrepancy in results obtained using 2 different normalization standards on identical samples stresses the necessity of using appropriate normalization strategy. Messenger RNA and microRNA were quantified by qPCR ($n \geq 6$). *$P < 0.05$ vs. ischemia in arbitrary units (AU) with ischemia = 1.

demonstrated at 15 min ischemic-reperfusion for miR-1: *GAPDH* 106.74 vs. U6B 74.82, and miR-101b: *GAPDH* 84.46 vs. U6B 81.35.

DISCUSSION

Our data demonstrate the importance of validating the normalization strategy when investigating miRNA expression. Moreover, several suitable normalization standards were identified for evaluation of miRNA expression in the ex vivo perfused rat hearts exposed to ischemia and reperfusion with the following ranking order: sno202 < U6B < U87 < snoRNA < 4.5S RNA A < Y1 < 4.5S RNA B < *GAPDH*. With U6B as the normalization standard, expression of miR-1 and miR-101b were significantly downregulated in the ischemic reperfused rat heart. Additionally, we describe a strategy for evaluating miRNA(s) of interest together with normalization standard(s) in one RT reaction.

Vandesompele et al. (28) recommend at least three normalization standards when performing qPCR on mRNAs, which also applies to ncRNAs. In kits like TaqMan MicroRNA assay (Applied Biosystems) the number of reference ncRNAs within each RT reaction is limited. However, a modification of the protocol supplied by the manufacturer allowed for inclusion of up to three ncRNAs in the same RT reaction by replacing RNase-free water with primers. Reverse transcription of three ncRNAs in each reaction, where one or two of the ncRNAs are verified references will allow a more precise normalization of ncRNA of interest. This was evaluated by investigating how the relationship between C_q values for miR-1, sno202, and U6B was affected after modulating the number of primers and total volumes compared with the standard protocol. According to Nolan et al. (19), C_q variation between reaction replicates should be <0.5. Hence, a comparison of replicates from modified and standard protocol of identical samples would give an indication of the applicability of modifications. Three primers in 15 μl reaction volume corresponded well to the original protocol (one primer in 15 μl reaction volume), being <0.5 C_q (Fig. 2). Further increase in volume and/or number of RT primers (30–45 μl and 3–9 primers) augmented the difference compared with standard protocol, with a variation of 1.0 or more. These observed differences indicate that modifications involving the latter should be avoided. However, using three RT primers in a standard reaction volume has a minor effect on C_q values. Hence, to reduce experimental error we recommend use of three primers per RT reaction represented by at least one normalization standard, which additionally will save time and costs.

There is no universally acceptable method for assessing expression stability of normalization standards, but software such as geNorm (28) and NormFinder (1) may simplify the selection of the most appropriate candidates. The Microsoft Excel application tool geNorm ranks candidates according to their expression stability by pair-wise comparison of the expression level of candidates analyzed (28), whereas NormFinder combine estimates of intra- and intergroup values to calculate a stability value for each normalization candidate analyzed (1). In the present study, equal rankings of candidates were obtained with sno202, U6B, and U87 being the top three candidates in both programs, respectively (Fig. 3). Generally, most reference ncRNAs tested here were stably expressed despite the fact that heart samples were collected at different time points at ischemic reperfusion (Fig. 1), corroborating their suitability as normalization standards in the ex vivo rat heart.

U6B is one of the most commonly used normalization standards for miRNA qPCR analysis despite lacking validation of this normalizer for all tissue types and experimental settings (8–10, 14). As mentioned, no ideal universal normalization standard exists. Hence, the suitability of U6B in the ex vivo ischemic rat heart does not necessarily apply to others as demonstrated by Peltier and Latham (20), who found U6B to be one of the least stable candidates in human cancerous tissue. This emphasizes the need to validate expressional stability of candidates for each experimental setting before choosing normalization standard.

The mRNA *GAPDH* was by far ranked as the least stable normalization candidate irrespective of qPCR assay used, hydrolysis probe or SYBR Green (Fig. 3). Even though the quantification assay of the mRNA is technically similar to the hydrolysis probe assay used for ncRNAs, the mRNA was ranked as the least stable normalization candidate, highlighting the difficulties of normalization against RNA of different "origins". The ranking of *GAPDH* may be explained by the fact that a normalization standard should mirror amount and size of the target of interest to obtain comparable isolation samples (20). MiRNAs represent a small fraction (~0.01%) of total RNA in a sample in addition to their small size (~22 nucleotides) (20), which contrasts to mRNAs representing ~5% of total RNA and may consist of thousands of nucleotides. Despite this, mRNAs such as *GAPDH* are still applied as reference genes in miRNA qPCR (11, 17, 21). Normalization standards that better reflect properties of miRNAs are other small ncRNAs such as snRNAs and snoRNAs. Even though these RNAs differ from miRNAs functionally, they resemble in size and are extracted from tissue by an identical assay approach.

The importance of choosing correct normalization strategy has been emphasized by several research groups as they demonstrated how results varied with the chosen reference gene in both mRNA (3–4, 12) and miRNA (20) qPCR experiments. However, results are still published without thorough validation of normalization strategy, which may lead to falsified detection of miRNA expression. To demonstrate, expression of miRNAs expected to be regulated at ischemia-reperfusion was evaluated when normalized to two of the most frequently used normalization standards, namely *GAPDH* (the least stable candidate), or to U6B (one of the more stably expressed candidates) (Fig. 4). The present study show a significant downregulation of miR-1 expression occurring at immediate reperfusion despite normalization standard applied (Fig. 4, *A* and *B*). Of importance, detecting significant regulation of miR-101b was dependent on the selected normalization candidate. When U6B was applied with a stable expression profile according to the stability ranking order obtained by the NormFinder and geNorm algorithms, miR-101b was significantly downregulated at 15 and 120 min of ischemic-reperfusion. On the contrary, if *GADPH* was applied as normalization standard, the expression level of miR-101b proved not to be regulated at ischemic reperfusion in the isolated ex vivo rat heart (Fig. 4, *C* and *D*), thus concealing regulation of this miRNA. Of note, these results (Fig. 4) only illustrate effect of normalization on downregulated miRNAs. However, the examination of stably

expressed normalization candidates were performed in a specific experimental model, and it is therefore reasonable to believe that the normalization procedure would be valid for any regulated miRNAs exposed to identical treatment (i.e., ischemic reperfusion in the isolated ex vivo rat heart). Regardless of this, Fig. 4 demonstrates that credible interpretation of results clearly depends on chosen normalization strategy, and a general rule of thumb should therefore always be to inspect normalization standards before use.

The results obtained in this study indicate that miR-1 and miR-101b is downregulated at immediate reperfusion in rat heart subjected to a lethal ischemic insult (1 and 15 min of reperfusion, respectively). Regulation is consistent with chronic studies where expression of these miRNAs has been examined hours to days after an AMI (18, 25–27). Further investigation is needed to delineate the exact role of miR-101b in the ischemic reperfused rat heart, but regarding the muscle-specific miRNA miR-1 there is mounting evidence for involvement in the developing heart and cardiovascular diseases such as AMI in experimental animals and humans (2, 25, 30).

In conclusion, several normalization candidates proved to be suitable for investigating miRNA expression in the isolated ex vivo rat heart, with sno202 being the most optimal candidate according to the NormFinder and geNorm algorithms. By a simple modification of the TaqMan MicroRNA assay (Applied Biosystems) it is possible to increase number of normalization standards in each RT reaction, and we recommend sno202 and U6B (and U87 if possible) to be included in the normalization process of cardiac miRNA expression analyses in the ischemic reperfused rat heart. Furthermore, these data imply that the miRNAs miR-1 and miR-101b are significantly regulated in the reperfused ischemic myocardium.

ACKNOWLEDGMENTS

Inger Ottesen is gratefully acknowledged for contribution in regards to isolation of total RNA.

Present address of T. Brattelid: National Institute of Nutrition and Seafood Research, Bergen, Norway.

GRANTS

This work was supported by The Research Council of Norway, Bergen Medical Research Foundation, The Meltzer Foundation, and University of Bergen Heart Foundation (to A. K. Jonassen) and by the Skipsreder Tom Wilhelmsen Foundation (to T. Brattelid).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Andersen CL, Jensen JL, Orntoft TF.** Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64: 5245–5250, 2004.
2. **Bostjancic E, Zidar N, Stajer D, Glavac D.** MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. *Cardiology* 115: 163–169, 2010.
3. **Brattelid T, Tveit K, Birkeland JA, Sjaastad I, Qvigstad E, Krobert KA, Hussain RI, Skomedal T, Osnes JB, Levy FO.** Expression of mRNA encoding G protein-coupled receptors involved in congestive heart failure–a quantitative RT-PCR study and the question of normalisation. *Basic Res Cardiol* 102: 198–208, 2007.
4. **Brattelid T, Winer LH, Levy FO, Liestol K, Sejersted OM, Andersson KB.** Reference gene alternatives to Gapdh in rodent and human heart failure gene expression studies. *BMC Mol Biol* 11: 22, 2010.
5. **Bustin SA.** Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25: 169–193, 2000.
6. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT.** The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55: 611–622, 2009.
7. **Cheng AM, Byrom MW, Shelton J, Ford LP.** Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 33: 1290–1297, 2005.
8. **Cheng Y, Ji R, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C.** MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? *Am J Pathol* 170: 1831–1840, 2007.
9. **Choong ML, Yang HH, McNiece I.** MicroRNA expression profiling during human cord blood-derived CD34 cell erythropoiesis. *Exp Hematol* 35: 551–564, 2007.
10. **Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY.** MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res* 67: 8433–8438, 2007.
11. **De Martino I, Visone R, Fedele M, Petrocca F, Palmieri D, Martinez Hoyos J, Forzati F, Croce CM, Fusco A.** Regulation of microRNA expression by HMGA1 proteins. *Oncogene* 28: 1432–1442, 2009.
12. **Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A.** The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal Biochem* 344: 141–143, 2005.
13. **Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin ES, Zhang C.** MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. *J Biol Chem* 284: 29514–29525, 2009.
14. **Fang Y, Shi C, Manduchi E, Civelek M, Davies PF.** MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro. *Proc Natl Acad Sci USA* 107: 13450–13455, 2010.
15. **Huggett J, Dheda K, Bustin S, Zumla A.** Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 6: 279–284, 2005.
16. **Jonassen AK, Sack MN, Mjos OD, Yellon DM.** Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 89: 1191–1198, 2001.
17. **Kim DH, Saetrom P, Snove O Jr, Rossi JJ.** MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA* 105: 16230–16235, 2008.
18. **Lu Y, Zhang Y, Shan H, Pan Z, Li X, Li B, Xu C, Zhang B, Zhang F, Dong D, Song W, Qiao G, Yang B.** MicroRNA-1 downregulation by propranolol in a rat model of myocardial infarction: a new mechanism for ischaemic cardioprotection. *Cardiovasc Res* 84: 434–441, 2009.
19. **Nolan T, Hands RE, Bustin SA.** Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 1: 1559–1582, 2006.
20. **Peltier HJ, Latham GJ.** Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 14: 844–852, 2008.
21. **Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R.** MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci USA* 105: 1608–1613, 2008.
22. **Qin W, Shi Y, Zhao B, Yao C, Jin L, Ma J, Jin Y.** miR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. *PLoS One* 5: e9429, 2010.
23. **Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A.** Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 313: 856–862, 2004.
24. **Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M.** MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ Res* 100: 416–424, 2007.
25. **Shan ZX, Lin QX, Fu YH, Deng CY, Zhou ZL, Zhu JN, Liu XY, Zhang YY, Li Y, Lin SG, Yu XY.** Upregulated expression of miR-1/miR-206 in a rat model of myocardial infarction. *Biochem Biophys Res Commun* 381: 597–601, 2009.
26. **Shi B, Guo Y, Wang J, Gao W.** Altered expression of microRNAs in the myocardium of rats with acute myocardial infarction. *BMC Cardiovasc Disord* 10: 11, 2010.

27. **van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN.** Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 105: 13027–13032, 2008.
28. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.
29. **Xu C, Lu Y, Pan Z, Chu W, Luo X, Lin H, Xiao J, Shan H, Wang Z, Yang B.** The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes. *J Cell Sci* 120: 3045–3052, 2007.
30. **Zhao Y, Samal E, Srivastava D.** Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 436: 214–220, 2005.