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Identification of cell-specific patterns of reference gene stability in quantitative reverse-transcriptase polymerase chain reaction studies of embryonic, placental and neural stem models of prenatal ethanol exposure

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Abstract

Identification of the transcriptional networks disrupted by prenatal ethanol exposure remains a core requirement to better understanding the molecular mechanisms of alcohol-induced teratogenesis. In this regard, quantitative reverse-transcriptase polymerase chain reaction (qPCR) has emerged as an essential technique in our efforts to characterize alterations in gene expression brought on by exposure to alcohol. However, many publications continue to report the utilization of inappropriate methods of qPCR normalization, and for many *in vitro* models, no consistent set of empirically tested normalization controls have been identified. In the present study, we sought to identify a group of candidate reference genes for use within studies of alcohol exposed embryonic, placental, and neurosphere stem cells under both conditions maintaining stemness as well as throughout *in vitro* differentiation. To this end, we surveyed the recent literature and compiled a short list of fourteen candidate genes commonly used as normalization controls in qPCR studies of gene expression. This list included: *Actb*, *B2m*, *Gapdh*, *Gusb*, *H2afz*, *Hk2*, *Hmbs*, *Hprt*, *Mrpl1*, *Pgk1*, *Ppia*, *Sdha*, *Tbp*, and *Ywhaz*. From these studies, we find no single candidate gene was consistently refractory to the influence of alcohol nor completely stable throughout *in vitro* differentiation. Accordingly, we propose normalizing qPCR measurements to the geometric mean C_T values obtained for three independent reference mRNAs as a reliable method to accurately interpret qPCR data and assess alterations in gene expression within alcohol treated cultures. Highlighting the importance of careful and empirical reference gene selection, the commonly used reference gene *Actb* was often amongst the least stable candidate genes tested. In fact, it would not serve as a valid normalization control in many cases. Data presented here will aid in the design of future experiments using stem cells to study the transcriptional processes driving differentiation, and model the developmental impact of teratogens.

Keywords

Reference gene; Normalization; Alcohol; Quantitative RT-PCR; Real-time PCR; Stem cells

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.alcohol.2012.12.003>.

Introduction

The American College of Obstetricians and Gynecologists has indicated that three out of every one hundred babies born in the United States exhibit one type of major birth defect. Of these, nearly one third are due to the consumption of alcohol during pregnancy (Cynthia, 1996; Floyd & Sidhu, 2004; Hoyme et al., 2005). As a result, 9.1 cases per 1000 live births exhibit some degree of fetal alcohol spectrum disorder, which can vary from barely detectable to severe functional and cognitive birth defects (Becker, Diaz-Granados, & Randall, 1996; Floyd, Weber, Denny, & O'Connor, 2009). Despite years of intense study, both the biochemical mechanisms of alcohol-induced teratogenesis and the developmental origins of fetal alcohol spectrum disorders remain poorly defined.

Identification of the transcriptional networks disrupted by prenatal ethanol exposure remains a core requirement to better understanding the molecular mechanisms of alcohol-induced teratogenesis. In this regard, the isolation and culture of both embryonic and tissue specific stem cells provide an enormous opportunity to model the molecular processes driving differentiation and study the developmental impact of teratogens. To gain insight into early embryonic development, pluripotent stem cells from both the embryonic and extraembryonic lineages present within the mammalian preimplantation blastocyst have been derived (Martin, 1981; Nagy, Rossant, Nagy, Abramow-Newerly, & Roder, 1993; Tanaka, Kunath, Hadjantonakis, Nagy, & Rossant, 1998). Embryonic (ES), and trophectoderm (TS) stem cells exhibit the developmental potential of these distinct cellular lineages and offer the opportunity to model early differentiation of the embryo and placenta respectively. Similarly, fetal neuroepithelial stem cells cultured as neurospheres enable the examination of the molecular mechanisms governing neurogenesis *in vitro*, and aid in our understanding of why, above all others, the brain is so profoundly affected by the consumption of alcohol during pregnancy (Frederiksen, Jat, Valtz, Levy, & McKay, 1988; McKay, 1997; Miranda, Santillano, Camarillo, & Dohrman, 2008; Taléns-Visconti et al., 2011). Using these unique cell types, researchers are now examining the transcriptional consequences of ethanol exposure and monitoring the efficacy of potential therapeutic interventions.

Quantitative reverse-transcriptase polymerase chain reaction (qPCR) has emerged as an essential technique in our efforts to characterize alterations in gene expression brought on by exposure to alcohol. Many publications, however, continue to report the utilization of inappropriate methods of data normalization, calling into question the conclusions put forward (Bustin et al., 2009). In studies of stem or progenitor cell differentiation, it has been well documented that changes of as little as two-fold can significantly alter the developmental program and change cell fate (Niwa, Miyazaki, & Smith, 2000). Given the recent evidence that alcohol has the capacity to alter cellular differentiation within the developing central nervous system (Santillano et al., 2005; Taléns-Visconti et al., 2011), reliable methods to identify the underlying transcriptional changes are crucial to deciphering the developmental origins of fetal alcohol spectrum disorders. For this reason, identifying a cohort of stable reference genes for use as normalization controls is absolutely essential for both accurate data interpretation and reliable candidate gene discovery. Despite the widespread use of qPCR in the field of alcohol research, to the best of our knowledge, only two studies have currently been published which consider reference gene stability within the context of alcohol exposure (Boujedidi et al., 2012; Johansson et al., 2007). The retractions of the 2005 Science breakthrough of the year and studies linking childhood vaccinations to autism are two recent, widely-publicized examples, which highlight the importance of qPCR data normalization to the integrity of the overall study (Böhlenius, Eriksson, Parcy, & Nilsson, 2007; Lancet, 2010). Empirical selection and validation of a set of stable reference

genes is absolutely essential to ensure both the validity of the measurements being taken as well as their accurate interpretation. To address recurring errors in qPCR data interpretation, Bustin and colleagues recently established the Minimum Information for publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines to ensure that studies utilizing qPCR are accurately interpreted and reproducible (Bustin et al., 2009). From these guidelines, the geometric mean of three carefully selected reference genes has become a validated requirement for normalizing qPCR data and the accurate assessment of quantitative changes in gene expression (Andersen, Jensen, & Ørntoft, 2004; Bustin et al., 2009; Gutierrez et al., 2008; Lanoix et al., 2012; Pfaffl, Tichopad, Prgomet, & Neuvians, 2004; Vandesompele et al., 2002).

Using these parameters, we sought to identify a list of candidate genes most suitable for use as normalization controls in qPCR-based comparisons between control and ethanol-exposed ES, TS, and neurosphere stem cells. In addition, we initiated studies to examine the stability of reference genes throughout the process of *in vitro* differentiation, and to identify the best possible reference genes to examine the impact of alcohol upon these processes. In order to help identify candidate genes, we set three main criteria that potential reference genes would have to fulfill: 1) the transcripts needed to be expressed above background and easily detectable, 2) candidate mRNAs needed to be expressed within each of the three cellular lineages under investigation, and 3) the genes needed to be expressed throughout *in vitro* differentiation. We then surveyed the recent literature and compiled a short list of fourteen candidate genes, including: Actb, B2m, Gapdh, Gusb, H2afz, Hk2, Hmbs, Hprt, Mrpl1, Pgk1, Ppia, Sdha, Tbp, and *Ywhaz* (Allen, Von Kaenel, Goodrich, & Kugel, 2004; Andersen et al., 2004; van den Bergen, Miles, Sinclair, & Western, 2009; Espinoza, Allen, Hieb, Kugel, & Goodrich, 2004; Galiveti, Rozhdestvensky, Brosius, Lehrach, & Konthur, 2010; Gilsbach, Kouta, Bönisch, & Brüss, 2006; Golding, Zhang, & Mann, 2010; Goossens et al., 2005; Gutierrez et al., 2008; Hwang, Wentzel, & Mendell, 2007; Mamo, Gal, Bodo, & Dinnyes, 2007; Pfaffl et al., 2004; Rugg-Gunn, Cox, Ralston, & Rossant, 2010; Suter, Tirefort, Julien, & Krause, 2009; Tatsumi et al., 2008; Veazey & Golding, 2011; Willems et al., 2006). These genes belong to diverse functional classes and should not be co-regulated in order to provide a non-biased method of normalizing qPCR expression data within ethanol-exposed cells (Supplemental Table S1). Results presented here identify the top three most stable reference genes suitable for normalization of qPCR-based studies of alcohol-induced teratogenesis within each of these three unique stem cell models, and highlight the importance of empirical reference gene selection.

Materials and methods

Embryonic and trophoblast stem cell culture

Previous studies in our laboratory have utilized stem cell lines derived from *Mus musculus castaneus* × *Mus musculus* (C57Black6) F1 embryos (Golding et al., 2010, 2011). Polymorphisms between these genetic strains allow the examination of mono-allelic patterns of epigenetic marks and gene expression within loci regulated by genomic imprinting (Golding et al., 2010). ES cultures were maintained in DMEM (Sigma, St. Louis, MO; Cat# D5671) supplemented with 50 µg/ml Penicillin/Streptomycin (Invitrogen, Carlsbad, CA; Cat# 15240096), 100 µM β-mercaptoethanol, 1× LIF (Sigma, St. Louis, MO; Cat# L5158), 2 mM L-Glutamine (Sigma, St. Louis, MO; Cat# G7513), 1× MEM non-essential amino acids (Invitrogen, Carlsbad, CA; Cat# 11140-050), and 15% Premium Select grade fetal bovine serum (Atlanta Biologicals Lawrenceville, GA; Cat# S11550). TS cell cultures were maintained as described (Golding et al., 2010; Tanaka et al., 1998) using RPMI (Sigma, St. Louis, MO; Cat# R0883) supplemented with 50 µg/ml Penicillin/Streptomycin, 1 mM Sodium Pyruvate (Invitrogen, Carlsbad, CA; Cat# 11360070), 100 µM β-mercaptoethanol, 1 µg/ml Heparin (Sigma, St. Louis, MO; Cat# H3149), 2 mM L-Glutamine, 1× FGF basic, 1×

FGF4 (R&D Systems, Minneapolis, MN; Cat# 233-FB and 235-F4 respectively), and 20% Premium Select FBS. Cells were initially grown on a Mytomycin C (Sigma, St. Louis, MO; Cat# M0503) treated feeder mouse fibroblast layer then moved to a feeder free system using conditioned medium as described previously (Tanaka et al., 1998).

For studies examining ES cell differentiation, a basic neuronal differentiation protocol was employed (Bain, Kitchens, Yao, Huettner, & Gottlieb, 1995). Briefly, sub-confluent ES cell cultures were lightly dissociated with 1× trypsin (Accutase – Millipore, Billerica, MA; Cat# SF006). Colonies were released from the plate but maintained as “clumps”. Dissociating colonies into individuals greatly reduced the number of cells surviving the differentiation procedure. Cellular clumps were plated in Corning ultra-low attachment flasks (VWR, Cat# 89089-876) using ES cell medium lacking LIF and β -mercaptoethanol, and cultured for four days. Subsequently, cells were treated with 0.5 M all-trans-retinoic acid (Sigma, St. Louis, MO; Cat# R2625) and cultured for an additional 4 days. Finally, cells were plated on 10 cm tissue culture treated dishes to differentiate into neuronal like cells. For studies examining ES cell differentiation and ethanol treatment, cells were cultured in medium containing 320 mg/dL ethanol and the lid was sealed with parafilm and placed in a standard incubator. Medium was changed every two days.

Neurosphere stem cell culture

Culture and media preparations for neurospheres have been described previously (Miranda et al., 2008). Briefly, fetal cerebral cortical neuroepithelial stem cells isolated from C57Black6 mice were cultured as neurospheres in T25 flasks in media containing GlutaMAX™ DMEM F-12 (Invitrogen, Carlsbad, CA; Cat# 11330-032), 20 ng/ml FGF basic, 20 ng/ml EGF (Invitrogen, Carlsbad, CA; Cat# 53003-018), 0.15 ng/ml LIF, 1× ITS-X (Invitrogen, Carlsbad, CA; Cat# 51500-056), 0.85 units/ml heparin, and 20 nM progesterone (Sigma, St. Louis, MO; Cat# P6149). Neurospheres were incubated for a total of 5 days at 37°C, 5% CO₂ in a humidified environment before passage. Medium was changed every 2–3 days depending on the level of confluence.

Differentiation of neurospheres

Neurospheres were grown in complete medium containing FGF, EGF, and LIF. Differentiation was initiated as previously described (Miranda et al., 2008). Briefly, neurosphere cultures were seeded onto Laminin (Invitrogen, Carlsbad, CA; Cat# 23017015) coated dishes to initiate dis-aggregation and extracellular matrix attachment of sphere-derived progenitor cells. After 2 days, EGF and LIF were removed from the medium. Within approximately 24 h, early neuronal differentiation was visualized (“SVZ” stage). The final cortical progenitor stage was achieved by removing FGF from the culture medium and culturing cells for an additional two days. At each stage, cells were collected and RNA extractions performed.

Ethanol treatment

Cells were cultured in T25 flasks, with a Parafilm (VWR) sealed lid to prevent ethanol evaporation. Dosing for ethanol followed previously published studies (Camarillo & Miranda, 2008), and utilized 60 mg/dL (13 mM), 120 mg/dL (26 mM), and 320 mg/dL (70 mM) of ethanol (Sigma). Control samples for non-ethanol treated stem cells were concurrently cultured during the same experimental time course and in the same culture conditions. Neurospheres received fresh medium every 2–3 days while the growth medium for ES and TS cell cultures were replenished every 48 h. On Day 5 of treatment, cells were collected and RNA extraction was performed.

RNA isolation and reverse transcription

Cultured cells were grown to 80% confluence, washed twice in warm PBS, and dissociated with 1× Trypsin (Accutase). Cells were spun down, washed once in cold PBS, and RNA isolated using Trizol (Invitrogen, Carlsbad, CA; Cat# 15596026) according to the manufacturer's protocol. 1 µg of purified total RNA was treated with amplification grade DNaseI (Sigma, St. Louis, MO; Cat# AMPD1) according to the manufacturer's protocol, and 250 ng of RNA was seeded into a reverse transcription reaction using the SuperScriptII kit (Invitrogen, Carlsbad, CA; Cat# 18064-071) by combining 1 µl random hexamer oligonucleotides (Invitrogen, Carlsbad, CA; Cat# 48190011), 1 µl 10 mM dNTP (Invitrogen, Carlsbad, CA; Cat# 18427-013), and 11 µl RNA plus water. This mixture was brought to 70°C for 5 min then cooled to room temperature. SuperScriptII reaction buffer, DTT, and SuperScriptII were then added according to manufacturer's protocol, and the mixture was brought to 25°C for 5 min, 42°C for 50 min, 45°C for 20 min, 50°C for 15 min, then 70°C for 5 min.

Real-time PCR amplification

Primers were designed using the NetPrimer software tool (www.premierbiosoft.com/netprimer) or identified from previously published research (Supplemental Table S2). Real-time PCR analysis of mRNA levels was carried out using the DyNAmo Flash SYBR Green qPCR Mastermix (Fisher Scientific, Pittsburgh, PA; Cat# F-415L) following the manufacturer's instructions. Reactions were performed on a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA).

Candidate gene ranking

Empirical selection and validation of a set of at least three stable reference genes has emerged as a core requirement for accurate interpretation of qPCR-based measurements of gene expression (Andersen et al., 2004; Bustin et al., 2009; Gutierrez et al., 2008; Pfaffl et al., 2004; Vandesompele et al., 2002). To facilitate the identification and selection of stable reference genes, three Microsoft Excel-based statistical programs have been produced and their predictive accuracy confirmed using published microarray data. GeNorm, NormFinder, and BestKeeper utilize slightly different methods of estimating both the intra- and the intergroup expression variation, and allow the ranking of candidate genes based on their overall stability across multiple experimental conditions and/or cell types. For example, GeNorm uses a measure of mean pair-wise variation between an individual candidate and the other reference genes measured to produce a stability value, and then ranks candidate genes according to this number (Vandesompele et al., 2002). NormFinder assigns a stability value based on statistical comparisons of inter- and intra-group stability and ranks candidate genes according to their expression variance across all samples measured (Andersen et al., 2004). Similarly, BestKeeper measures the geometric mean for each experiment, computes a pair-wise correlation coefficient for each candidate and then ranks candidate genes according to this index (Pfaffl et al., 2004). As BestKeeper has a maximal allowance of ten candidate genes, we elected to exclude this software package from our analyses. In each of our experiments we have tabulated the rankings from both the GeNorm and Normfinder software tools. Candidate genes with GeNorm stability values greater than 0.5 are not acceptable for use as normalization controls, and have been demarcated with a superscript letter 'a' (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). A similar threshold for Normfinder has not been demonstrated, therefore only the rankings can be utilized as a guide for candidate gene suitability. Each of these programs is publicly available as a macro-program, which runs in older versions of Microsoft Excel.

Analysis of real-time PCR data and statistical analyses

The measured C_T (Cycle Threshold) values for each sample were compiled and the stability of each of the fourteen reference genes analyzed using the GeNorm and NormFinder software tools (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002). Once suitable reference genes were identified, the geometric mean C_T values of the best three candidate genes were calculated for each individual sample and used to normalize expression levels using the $\Delta\Delta C_T$ method described previously (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008; Vandesompele et al., 2002). Normalized C_T values were averaged and the standard error of the mean calculated and graphed using Excel.

Using the JMP (SAS Institute, NC) software package, we conducted one- and two-way ANOVAs, as appropriate, and applied Tukey's HSD analysis for multiple comparisons. Main effects and first order interactions were considered in all models.

Results

Reference gene stability in embryonic stem cells

We began by examining the stability of our candidate reference genes in mouse ES cells exposed to varying concentrations of ethanol (EtOH). Based on previous studies, we chose to examine 60 mg/dL, 120 mg/dL, and 320 mg/dL exposures of ethanol (Camarillo & Miranda, 2008). Unsurprisingly, cells cultured in the highest concentration of EtOH displayed slower growth and increased amounts of cellular debris suggesting a rise in the number of dead or dying cells. However, cells cultured in 120 mg/dL did display a modest increase in growth rate that trended toward statistical significance, but did not achieve a p -value less than 0.05 (data not shown). Cell lines were cultured to 80% confluence under both control and ethanol conditions, cellular extracts collected, and RNA isolated from four independent experimental replicates ($N=4$). RNA from each replicate was seeded into four independent reverse transcription reactions and used in three independent qPCR reactions measuring the fourteen candidate genes in duplicate. From these experiments, the C_T values were compiled and analyzed using the GeNorm and NormFinder software packages (Andersen et al., 2004; Vandesompele et al., 2002). Results from these analyses consistently identified *Gapdh*, *Ppia*, and *Hprt* as the most stable reference genes across all experimental treatments examined. In contrast, *Pgk1* and *Gusb* displayed GeNorm stability values in excess of 0.5, indicating they would not serve as acceptable normalization controls (Table 1a) (Hellemans et al., 2007). To visualize changes in candidate gene expression in response to our experimental treatments, we utilized the geometric mean of these three best candidates to normalize and graph our qPCR data using the comparative or $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). While treatment with EtOH increased variability, it did not dramatically alter the expression of any one gene (Fig. 1A). While *Actb* did display a 1.5–2-fold change across all EtOH treatments, a one-way ANOVA revealed this change was not statistically significant. Moreover, this analysis revealed that exposure to alcohol did not significantly influence the expression of any of the candidate genes examined.

The *in vitro* differentiation of embryonic and tissue specific stem cells provides an enormous opportunity to model the cellular processes driving differentiation, and allows mechanistic studies into the molecular actions of teratogens. We next sought to determine the influence of EtOH upon reference gene stability throughout the process of *in vitro* differentiation. ES cells were differentiated using a standard embryoid-body-neuronal differentiation protocol (see Materials and Methods section). ES cells were gently dissociated into large clumps, transferred to ultra-low adherence flasks and exposed to retinoic acid (see Materials and Methods section). For these experiments, we focused on comparisons between control and

320 mg/dL EtOH treatments. Cells from both groups readily formed embryoid bodies and neurons when plated under appropriate conditions. Cells within the 320 mg/dL EtOH treated cultures exhibited a large increase in the amount of cellular debris, consistent with previous studies (Arzumnyan, Anni, Rubin, & Rubin, 2009), demonstrating increased apoptosis within differentiating ES cell cultures exposed to alcohol. Samples were collected on Days 3, 6, and 9 of the differentiation protocol and RNA was analyzed as previously described. From this analysis, *Ppia*, *Sdha*, and *Mrp11* emerged as the most consistently stable reference genes (Table 1b). Interestingly, the commonly used reference gene *Gapdh* yielded a stability value greater than 0.5, indicating this nearly ubiquitous reference gene is a poor choice for normalizing qPCR expression data when examining the consequences of ethanol exposure during ES cell differentiation. We then utilized the geometric mean of the three highest ranked candidate genes to normalize and graph the relative expression of our fourteen reference genes (Fig. 1B and C). Analysis using a two-way ANOVA considering the two experimental treatments across days of the differentiation protocol revealed exposure to ethanol did not significantly impact the expression of any of our candidate genes. As a means to further validate our normalization controls, we utilized the top three reference genes as a base to monitor the expression of the well-established ES cell pluripotency markers *Oct4*, *Nanog*, and *Klf4* throughout ES cell differentiation. Changes in the expression of these transcription factors throughout *in vitro* differentiation are extremely well characterized, offering the opportunity to confirm the validity of our chosen references. As expected, each of these markers demonstrated coordinated down-regulation throughout the course of ES cell differentiation (Fig. 2). We conducted a two-way ANOVA, including interactions, comparing ethanol treatments across the nine-day experimental course to determine if EtOH induced alterations in the expression of these transcription factors. Although significant differences were detected, Tukey's HSD analysis revealed that these were due to the differentiation that occurred across days in culture and that EtOH did not significantly influence the expression of our candidate pluripotency markers (Fig. 2).

Reference gene stability in trophectoderm stem cells

To model the impact of ethanol exposure on placentation, we examined the consequences of exposing trophectoderm stem (TS) cells *in vitro* to varying concentrations of EtOH. For these experiments we utilized control, 120 mg/dL and 320 mg/dL EtOH treatments. Surprisingly, TS cells did not exhibit any noticeable changes in growth rate nor an increase in cell death, even when exposed to the higher range of EtOH concentrations tested. Cells were cultured to 80% confluence, RNA isolated and analyzed as above. From these analyses, all of the fourteen candidate genes examined would serve as suitable normalization controls (Table 2a). When we normalized gene expression levels using the geometric mean of the top three candidate genes (*Sdha*, *Hprt*, and *Mrp11*), we found the expression levels of all candidate genes to be completely homogenous (Fig. 3A). A one-way ANOVA comparing all experimental treatments for each gene confirmed no statistically significant changes in gene expression could be detected.

To examine the influence of EtOH on the stability of our candidate reference genes throughout TS cell differentiation, we followed a previously described differentiation protocol (Tanaka et al., 1998; Veazey & Golding, 2011). For these experiments, we focused on comparisons between 120 mg/dL EtOH, 320 mg/dL EtOH, and control treatments. Twenty-four hours after plating, alcohol was added to the newly seeded TS cell cultures. Twenty-four hours later, FGF4 and heparin were withdrawn from the culture medium, marking Day 0 of the differentiation protocol. Over the course of the next five days, TS cell cultures readily differentiated into placental giant cells. In contrast to our analysis of undifferentiated TS cells, however, the 320 mg/dL treatments produced a modest increase in cell death as measured solely by increased cellular debris. RNA was then isolated and

analyzed as described in the Materials and Methods section. Interestingly, TS cells again showed a very narrow range of reference gene fluctuation, with *Sdha*, *Mrpl1* and *Ppia* being ranked as the three most stable genes (Table 2b). Using the geometric mean of *Sdha*, *Ppia*, and *Mrpl1*, we normalized and graphed the relative expression of our fourteen candidate genes (Fig. 3B). Similar to previously published observations, *Actb* exhibited large fluctuations in expression inherent to TS cell differentiation (Veazey & Golding, 2011; Vong et al., 2010). Using a two-way ANOVA examining experimental treatments across days in culture, we did not find any significant alterations in gene expression arising as a consequence of EtOH exposure. To validate our normalization controls, we utilized the geometric mean of *Sdha*, *Ppia*, and *Mrpl1* to graph the expression of the TS cell stemness marker *Cdx2*. Similar to our analyses of *Oct4*, *Nanog*, and *Klf4* in differentiating ES cells, we observed a large decrease in the expression of this cellular marker of trophoctoderm stem cell potency throughout the course of *in vitro* differentiation (Fig. 3C). To determine if EtOH induced alterations in the expression of *Cdx2*, we conducted a two-way ANOVA comparing ethanol doses over time. This analysis revealed that on Day 0, the 320 mg/dL treatments significantly reduced *Cdx2* expression, whereas, the 120 mg/dL treatments remained unaffected and were identical to the control ($p < .0001$).

Reference gene stability in neurosphere stem cells

Fetal development is characterized by multiple region-specific periods of neurogenic growth, giving rise to millions of neuroblasts that migrate away from their germinal zones to populate the developing brain. To model this developmental process *in vitro*, cell cultures have been derived from the fetal neuroepithelium. These stem cells grow as free-floating, clonal aggregates termed “neuro-spheres” (Conti & Cattaneo, 2010). To determine the impact of EtOH exposure on our fourteen candidate reference genes within this model system, neurospheres were cultured under control conditions or 60 mg/dL, 120 mg/dL, or 320 mg/dL EtOH and analyzed as above. As can be seen in Table 3a, *Actb* stability is affected by exposure to EtOH, and would not serve as an acceptable normalization control. From these experiments, the top three candidate genes identified were *Pgk1*, *Gapdh*, and *Hprt*. Using the geometric mean of these three candidates as normalization controls, we graphed the relative expression levels of the fourteen candidate genes (Fig. 4A). Using a one-way ANOVA comparing each experimental treatment, we were unable to discern any significant differences between the treatment groups, for any of the candidate genes examined.

We next sought to examine the impact of ethanol exposure on neurosphere differentiation. We began by examining the expression of our candidate genes throughout a previously published, five-day neurosphere differentiation protocol (Miranda et al., 2008). Candidate gene expression was assayed during the early and late stages of differentiation. From these analyses, we observed that the expression of many of our reference genes were profoundly altered during the course of neurosphere differentiation, with six of the fourteen genes having GeNorm stability values in excess of 0.5. As can be seen in Table 3b, *Hprt*, *Sdha*, *Mrpl1*, and *Gusb* were identified as the most consistently stable reference genes. Using the geometric mean of *Hprt*, *Mrpl1*, and *Sdha*, we normalized and graphed the relative expression of the fourteen candidate genes using methods previously discussed (Fig. 4B). Using a two-way ANOVA comparing experimental treatments across days of the differentiation protocol, we observed statistically significant differences in the expression of *Gapdh*, *H2afz*, *Tbp*, and *Ywhaz*.

Unexpectedly, when we assayed the expression of mRNAs encoding markers of neurosphere stemness (*Sox2* and *Nestin*), we found that their expression significantly increased as differentiation progressed (Fig. 5). Paradoxically, while these markers of stemness increased so did established makers of neuronal differentiation, including *Gfap* and

Dlx2, *Gfap*, which was minimally detected on Day 0, became undetectable on Day 3 and strongly expressed on Day 5. In contrast, *Dlx2* was undetectable on Day 0 but progressively increased over the course of *in vitro* differentiation. Similar to previous reports, the relative ratios in which these transcription factors were expressed seemed to change through cellular passaging (Conti & Cattaneo, 2010). This suggests the complex population of cells present within neurospheres was constantly fluctuating. From previous studies (Campos, 2004), it has been suggested that the heterogeneity of the neurospheres is linked to their three-dimensional structure, where different cells within the spherical structure are exposed to varying and sometimes suboptimal conditions. For example, neurospheres display a tendency to generate differentiated cells within their core while cells on the surface are more stem-like (Campos, 2004). These results prompted us to question what proportions of the cellular population were differentiating versus those that were being maintained in a stem-like-state simply due to either exposure to local paracrine factors or cell–extracellular matrix interactions. Most studies of neurosphere differentiation predominately utilize immunocytochemistry to visually examine the expression of cellular markers within individual populations of cells. These studies tend to focus on individual colonies of cells rather than the total population of cells as in qPCR studies. Our results suggest a global survey of the transcriptome using qPCR may not be sensitive enough to finely monitor the intricacies of gene expression during neurosphere differentiation. Similar to previous studies (Camarillo & Miranda, 2008; Santillano et al., 2005), under conditions of alcohol exposure we observed an increase in differentiating neurosphere size, and many of the cultures exhibited drastic differences in cellular morphology or progression through the stages of differentiation. Due to the extreme variation in both cell type and the expression of molecular markers within these cultures, we did not feel we were able to definitively and reliably assay candidate gene stability through differentiation.

Discussion

Analysis of gene expression using quantitative reverse-transcriptase polymerase chain reaction (qPCR) has become a widely utilized core technique in nearly every area of biological research. However, many publications continue to report the implementation of inappropriate methods of qPCR normalization (Bustin et al., 2009). A recent study of 8-Oxoguanine DNA glycosylase expression in preeclamptic placental samples by Lanoix et al., elegantly demonstrates how normalization with an inappropriate reference gene can produce a statistically significant result, which is diametrically opposite to the one predicted of a characterized physiological response (Lanoix et al., 2012). Despite numerous publications demonstrating significant fluctuations in the expression of established reference genes such as *Actb* and *Gapdh*, the single use of either of these reference genes to normalize gene expression data remains the norm for the vast majority of published qPCR-based studies (Bustin & Nolan, 2004; Dheda et al., 2005; Goossens et al., 2005; Johansson et al., 2007; Mamo et al., 2007; Radoni et al., 2004; Vandesompele et al., 2002). While many *in vitro* and *in vivo* studies modeling prenatal alcohol exposure employ qPCR-based measurements as a key analytic tool, very few studies have been published which consider the suitability/stability of their reference gene(s) (Boujedidi et al., 2012; Johansson et al., 2007).

Previous studies by Vandesompele and colleagues demonstrated that the geometric mean of three reference genes provided the most accurate and reliable means of normalizing qPCR expression data (Vandesompele et al., 2002). Subsequently, this experimental strategy was validated, and a series of statistical algorithms was written in order to identify the three most suitable reference genes for a variety of experimental conditions (Andersen et al., 2004; van den Bergen et al., 2009; Boda, Pini, Hoxha, Parolisi, & Tempia, 2009; Galiveti et al., 2010; Gilsbach et al., 2006; Goossens et al., 2005; Gutierrez et al., 2008; Pfaffl et al., 2004; Suter

et al., 2009; Tatsumi et al., 2008; Veazey & Golding, 2011; Willems et al., 2006). The GeNorm software tool ranks candidate genes according to a derived M-value (Vandesompele et al., 2002). An M-value describes the variation of a gene relative to all the other candidate genes examined. The GeNorm algorithm progressively assigns M-values by removing the gene with the highest M-value, and repeating the process until only the optimal set of reference genes remains (Vandesompele et al., 2002). In contrast, Normfinder was designed to independently account for the various sample or treatment groups (Andersen et al., 2004). This allows for the selection of an optimum reference gene pair rather than ranking genes individually from highest to lowest stability. Using this slightly different approach, the most stable pair of genes selected by Normfinder may have compensating expression levels. For instance, one reference gene may be highly expressed in one sample group while its paired gene is underexpressed in the same group. This difference between algorithms explains the slight differences in the assigned stability values and gene rankings observed in this study. Many of the gene rankings reported, however, were only different by a very small margin or were in fact the same but grouped differently due to alphabetical ordering of the gene names.

In the present study, we sought to assay the stability of fourteen commonly used reference genes within *in vitro* cultures of embryonic, placental, and neurosphere stem cells exposed to alcohol. Using these two independent software tools to assess gene stability, we find broad consensus in the identification of the three best normalization controls across cell types, alcohol exposures, and stages of *in vitro* differentiation. Based upon the framework provided by MIQE Guidelines, we propose utilizing the geometric mean of the top three genes identified for each experimental condition as normalization controls in studies examining the impact of alcohol upon the transcriptomes of the three stem cell types examined.

The overarching goal of this paper was to lay the groundwork for the use of embryonic, trophoblast and neurosphere stem cells as models to examine the transcriptional consequences of prenatal ethanol exposure. While our study found no single reference gene to be entirely refractory to the influence of alcohol nor completely stable throughout *in vitro* differentiation, *Hprt*, *Mrpl1*, *Sdha*, *Ppia* (and to a lesser extent *Gapdh*), were identified as the most consistently stable transcripts across all cell types and experimental conditions examined. This core group of candidates should be considered as a starting point for future studies of reference gene stability within other models of alcohol exposure.

In contrast, our analyses identified transcripts encoding one of the most commonly used reference genes, *Actb*, as often amongst the least stable candidate genes tested, and in many cases, it would not serve as an acceptable normalization control. Importantly, a significant number of studies modeling prenatal alcohol exposure continue to utilize *Actb* as their sole normalization control. Given the observations reported here and elsewhere indicating alcohol can modulate expression of this gene (Romero et al., 2010), its candidacy as a valid reference should be reconsidered. Overall, these investigations highlight the importance of empirical reference gene selection and identify a core group of candidate genes for use as normalization controls in qPCR studies of ethanol induced teratogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Analysis of reference genes in ethanol-exposed embryonic stem cells. A) Experiments contrasting control samples with 60 mg/dL, 120 mg/dL, and 320 mg/dL ethanol treatments under conditions maintaining ES cell stemness. Data were normalized to the geometric mean of *Gapdh*, *Ppia*, and *Hprt*. Using a one-way ANOVA, no statistically significant differences in candidate gene expression were observed ($p < .05$). Graphs are representative of four independent cell culture experiments ($N = 4$). B) & C) Relative expression of the fourteen candidate reference genes in differentiating embryonic stem cell cultures contrasting control samples with 320 mg/dL ethanol treatments. Samples were collected on Days 0, 3, 6, and 9 of a standard neuronal differentiation protocol. Data were normalized to the geometric mean of *Mrpl1*, *Ppia*, and *Sdhb*. Figures 1B and 1C were grouped and separated due to differences in the scale of candidate gene expression level. A two-way ANOVA revealed exposure to alcohol did not influence the expression of any of the candidate genes examined ($p < .05$). Graphs are representative of three independent cell culture experiments ($N = 3$). All data presented in this figure were graphed as relative values using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Error bars represent the standard error of the mean.

Fig. 2.

Validation of normalization controls in ES cell cultures. Relative expression of stem cell markers of pluripotency *Oct4*, *Nanog*, and *Klf4* in differentiating ES cells under either control conditions or exposure to 320 mg/dL ethanol. Data were normalized to the geometric mean of *Mrp11*, *Ppia*, and *Sdha*. Tukey's HSD analysis revealed the significant differences observed ($p < .0001$) were only across days in culture, and that EtOH did not significantly influence the expression of the candidate pluripotency markers. All data presented in this figure were graphed as relative values using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Graphs are representative of three independent cell culture experiments ($N = 3$). Error bars represent the standard error of the mean.

Fig. 3.

Analysis of reference genes in ethanol-exposed trophectoderm stem cells. A) Relative expression of the fourteen candidate reference genes in trophectoderm stem cell cultures contrasting control samples with 120 mg/dL and 320 mg/dL ethanol treatments. Cells were cultured under conditions maintaining TS cell stemness. Data were normalized to the geometric mean of *Sdha*, *Hprt*, and *Mpl1*. Using a one-way ANOVA, no statistically significant differences in candidate gene expression were observed ($p < .05$). Graphs are representative of four independent cell culture experiments ($N = 4$). B) Relative expression of the fourteen candidate reference genes in differentiating trophectoderm stem cell cultures contrasting control samples with 120 mg/dL and 320 mg/dL ethanol treatments. Samples were collected on Days 0 and 5 of *in vitro* differentiation. Data were normalized to the geometric mean of *Mpl1*, *Ppia*, and *Sdha*. Using a two-way ANOVA, no statistically significant differences in candidate gene expression could be detected ($p < .05$). Graphs are representative of four independent cell culture experiments ($N = 4$). C) Validation of normalization controls in differentiating TS cell cultures. Expression of the TS cell marker of pluripotency, *Cdx2*, in differentiating TS cells under either control conditions or exposure to 120 mg/dL or 320 mg/dL ethanol. *Cdx2* expression was normalized to the geometric mean of *Mpl1*, *Ppia*, and *Sdha*. A two-way ANOVA and Tukey's HSD analysis comparing ethanol doses over time revealed *Cdx2* levels within the Day 0 320 mg/dL EtOH treatments were significantly different than the Day 0 120 mg/dL and control treatments ($p < .0001$). No significant differences were found for the Day 5 cultures. Graphs are representative of four independent cell culture experiments ($N = 4$). All data presented in this figure were graphed as relative values using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Error bars represent the standard error of the mean.

Fig. 4.

Analysis of candidate reference genes in neurosphere stem cells. A) Relative expression of the fourteen candidate reference genes in neurosphere stem cell cultures contrasting control samples with 60 mg/dL, 120 mg/dL, and 320 mg/dL ethanol treatments. Data were normalized to the geometric mean of *Pgk1*, *Hprt*, and *Gapdh*. Using a one-way ANOVA, no statistically significant differences in candidate gene expression were observed ($p < .05$). Graphs are representative of four independent cell culture experiments ($N = 4$). B) Relative expression of the fourteen candidate reference genes in differentiating neurosphere cultures. Samples were collected on Days 0, 3, and 5 of the *in vitro* differentiation protocol as previously described (Miranda et al., 2008). Data were normalized to the geometric mean of *Hprt*, *Mrpl1*, and *Sdha*. Statistically significant differences in the expression of *Gapdh*, *H2afz*, *Tbp* and *Ywhaz* were identified using a two-way ANOVA followed by Tukey's HSD analysis. Graphs are representative of three independent cell culture experiments ($N = 3$). All data presented in this figure were graphed as relative values using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Error bars represent the standard error of the mean.

Fig. 5.

Analysis of lineage specific transcription factors in differentiating neurosphere stem cell cultures. Expression of neurosphere stem cell markers of stemness (*Sox2* and *Nestin*) as well as differentiation (*Gfap* and *Dlx2*) in differentiating neurosphere stem cells under control conditions. Expression was normalized to the geometric mean of *Hprt*, *Mrpl1*, and *Sdha*. Graphs are representative of three independent cell culture experiments ($N = 3$). Statistically significant differences were observed for all four candidate genes examined using either an ANOVA (*Sox2* and *Nestin*) or a student's *t*-test (*Dlx2* *Gfap*). All data presented in this figure were graphed as relative values using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Error bars represent the standard error of the mean.

Table 1

GeNorm and Normfinder rankings of candidate gene stability in experiments comparing ethanol and control treatments of embryonic stem cell cultures.

Rank	GeNorm		Normfinder	
	Gene name	Stability value	Gene name	Stability value
<i>a. Cultures maintained as stem cells</i>				
1	Gapdh	0.092	Gapdh	0.019
2	Ppia	0.104	Ppia	0.033
3	Hprt	0.107	Hprt	0.045
4	Ywhaz	0.107	Ywhaz	0.048
5	B2m	0.115	H2afz	0.05
6	Mrpl1	0.115	B2m	0.054
7	H2afz	0.116	Mrpl1	0.058
8	Actb	0.12	Actb	0.06
9	Sdha	0.122	Sdha	0.06
10	Tbp	0.341	Hmbs	0.074
11	Hmbs	0.373	Tbp	0.078
12	Hk2	0.424	Hk2	0.083
13	Pgk1 ^a	0.577	Pgk1	0.095
14	Gusb ^a	0.678	Gusb	0.102
<i>b. Differentiating cultures</i>				
1	Ppia	0.088	Ppia	0.027
2	Sdha	0.095	Mrpl1	0.037
3	Mrpl1	0.096	Sdha	0.04
4	Hk2	0.098	Hk2	0.043
5	Hprt	0.1	Ywhaz	0.044
6	Gusb	0.102	Gusb	0.045
7	Ywhaz	0.103	Hprt	0.045
8	Hmbs	0.104	Hmbs	0.047
9	B2m	0.105	Tbp	0.048
10	Tbp	0.106	B2m	0.051
11	H2afz	0.151	H2afz	0.059
12	Actb	0.187	Actb	0.064
13	Gapdh ^a	0.545	Gapdh	0.086
14	Pgk1 ^a	0.681	Pgk1	0.106

^a Candidate genes with GeNorm stability values greater than 0.5 are not acceptable for use as normalization controls (Hellemans et al., 2007).

Table 2

GeNorm and Normfinder rankings of candidate gene stability in experiments comparing ethanol and control treatments of trophectoderm stem cell cultures.

Rank	GeNorm		Normfinder	
	Gene name	Stability value	Gene name	Stability value
<i>a. Cultures maintained as stem cells</i>				
1	Hprt	0.028	Sdha	0.002
2	Ppia	0.028	Hprt	0.003
3	Sdha	0.028	Mrpl1	0.003
4	Ywhaz	0.028	Ppia	0.003
5	Mrpl1	0.029	Ywhaz	0.006
6	Gapdh	0.03	Pgk1	0.007
7	Pgk1	0.03	Gapdh	0.008
8	Actb	0.031	Actb	0.01
9	Tbp	0.035	Tbp	0.014
10	B2m	0.037	B2m	0.015
11	H2afz	0.043	H2afz	0.023
12	Hk2	0.054	Hk2	0.031
13	Gusb	0.068	Gusb	0.043
14	Hmbs	0.101	Hmbs	0.068
<i>b. Differentiating cultures</i>				
1	Sdha	0.048	Sdha	0.009
2	Mrpl1	0.049	Ppia	0.01
3	Gapdh	0.05	Mrpl1	0.013
4	Ppia	0.05	Gapdh	0.016
5	B2m	0.057	B2m	0.023
6	Ywhaz	0.061	H2afz	0.03
7	Hprt	0.062	Ywhaz	0.03
8	Pgk1	0.062	Hprt	0.031
9	H2afz	0.063	Pgk1	0.031
10	Gusb	0.07	Gusb	0.037
11	Hk2	0.072	Hk2	0.038
12	Hmbs	0.08	Hmbs	0.046
13	Tbp	0.081	Tbp	0.05
14	Actb	0.094	Actb	0.058

Table 3

GeNorm and Normfinder rankings of candidate gene stability in experiments comparing ethanol and control treatments of neurosphere stem cell cultures.

Rank	GeNorm		Normfinder	
	Gene name	Stability value	Gene name	Stability value
<i>a. Cultures maintained as stem cells</i>				
1	Gapdh	0.078	Pgk1	0.495
2	Pgk1	0.079	Gapdh	0.52
3	Tbp	0.079	Hprt	0.595
4	Hprt	0.081	Sdha	0.622
5	Sdha	0.081	Tbp	0.627
6	Gusb	0.084	Ywhaz	0.692
7	H2afz	0.086	H2afz	0.737
8	Mrpl1	0.086	Mrpl1	0.786
9	Ywhaz	0.088	Gusb	0.805
10	B2m	0.094	B2m	0.945
11	Ppia	0.111	Ppia	1.405
12	Hmbs	0.325	Actb	1.707
13	Hk2	0.434	Hmbs	1.859
14	Actb ^a	0.869	Hk2	2.397
<i>b. Differentiating cultures</i>				
1	Hprt	0.063	Hprt	0.012
2	Sdha	0.064	Sdha	0.016
3	Gusb	0.065	Mrpl1	0.017
4	Mrpl1	0.065	Gusb	0.018
5	Gapdh	0.067	Gapdh	0.023
6	Pgk1	0.068	Pgk1	0.023
7	Hk2	0.107	Hk2	0.026
8	Ywhaz	0.479	Hmbs	0.038
9	Hmbs ^a	0.803	Ywhaz	0.038
10	Tbp ^a	0.833	Ppia	0.042
11	Ppia ^a	0.857	Tbp	0.042
12	H2afz ^a	0.901	H2afz	0.059
13	B2m ^a	0.921	B2m	0.075
14	Actb ^a	1.544	Actb	0.1

^aCandidate genes with GeNorm stability values greater than 0.5 are not acceptable for use as normalization controls (Hellemans et al., 2007).