Establishing reference genes for use in real-time quantitative PCR analysis of early equine embryos

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Short title: **Reference genes for qPCR in early equine embryos.**

Abstract

Real-time quantitative PCR (qPCR) is invaluable for investigating changes in gene expression during early development, since it can be performed on the limited quantities of mRNA contained in individual embryos. However, the reliability of this method depends on the use of validated stably expressed "reference genes" for accurate data normalization. The aim of this study was to identify and validate a set of reference genes suitable for studying gene expression during equine embryo development. The stable expression of 4 carefully selected reference genes and 1 developmentally regulated gene was examined by qPCR in equine morula to expanded blastocyst stage *in vivo* embryos. *SRP14, RPL4* and *PGK1* were identified by geNorm analysis as stably expressed reference genes suitable for data normalization. *RPL13A* expression was less stable and changed significantly during the period of development examined, rendering it unsuitable as a reference gene. As anticipated, *CDX2* expression increased significantly during embryo development supporting its possible role in trophectoderm specification in the horse. In summary, we demonstrated that evidence-based selection of potential reference genes can reduce the number needed to validate stable expression in an experimental system; this is particularly useful when dealing with tissues that yield small amounts of mRNA. *SRP14*, *RPL4* and *PGK1* are stable reference genes suitable for normalizing expression for genes of interest during *in vivo* morula to expanded blastocyst development of horse embryos.

Introduction

In eutherian mammals, pre-implantation embryo development is a period of dynamic transition, spanning a range of important events that include cell cleavage, embryonic genome activation, cell lineage segregation, blastocyst formation, initial interaction with the endometrium and ultimately implantation. Large fluctuations in the transcriptome occur during this period, as a result of the switch from maternally-produced to embryo-produced

mRNA transcripts and an increase in the expression of growth, differentiation and transcription factors (Telford *et al.* 1990; Hamatani *et al.* 2004). Examining the expression of genes involved in these critical developmental processes is a more sensitive way of assessing the health and normal development of embryos than morphological criteria alone. This understanding may be invaluable for identifying causes of early embryonic loss, and in assisting the development of safe and effective *in vitro* assisted reproductive technologies.

In the horse, the embryonic genome becomes transcriptionally active by the third cleavage stage (5 to 8 cells) at approximately 72 h after fertilization (Brinsko *et al.* 1995; Grondahl and Hyttel 1996). However, the early horse embryo remains for an unusually long period (approximately 6 days) in the oviduct, making access to *in vivo* cleavage stages impossible without invasive surgery or slaughter (Betteridge 2007). When the embryo finally enters the uterus on day 6–6.5 it is usually at the compacted morula stage with a thick zona pellucida, but develops into an early blastocyst with the first visible signs of trophectoderm versus inner cell mass (ICM) differentiation within a few hours (Battut *et al.* 1997; Betteridge 2007). During initial intra-uterine development, the zona pellucida thins before being shed to leave an expanded blastocyst at around day 7–8 that is completely surrounded by a unique glycoprotein tertiary embryo coat, the blastocyst 'capsule', that had formed between the trophectoderm and the zona pellucida (Tremoleda *et al.* 2003; Stout *et al.* 2005). The capsule remains until approximately day 21 of gestation and appears to be essential for the establishment and maintenance of pregnancy (Betteridge *et al.* 1982; Stout *et al.* 2005).

Morula to expanded blastocyst stage equine embryos are of practical interest because these stages are used commercially for embryo transfer and cryopreservation; and are the stages to which *in vitro* produced or cloned embryos are cultured prior to transfer to the uterus of a mare (Stout 2006). In addition, these are the earliest *in vivo* developmental stages that can be

obtained without surgical intervention. The morula to blastocyst transition also spans important developmental events including: (i) the first (ICM vs. trophectoderm) and second (epiblast vs. hypoblast) cell lineage segregation events (Ralston and Rossant 2005; Kuijk *et al.* 2008; Harvey *et al.* 2009); (ii) formation of the blastocyst capsule (Stout *et al.* 2005); and (iii) rapid embryonic expansion and an increase in cell number from around 160 to over 2,900 (Tremoleda *et al.* 2003; Rambags *et al.* 2005). Moreover, this is a critical period for assisted reproductive technologies in the horse because: (i) embryonic stem cells are usually isolated from the ICM (Saito *et al.* 2002; Li *et al.* 2006); (ii) embryos >300 μm show much poorer survival following cryopreservation than smaller embryos (Slade *et al.* 1985; Tharasanit *et al.* 2005); and (iii) capsule formation is abnormal in *in vitro* produced embryos (Tremoleda *et al.* 2003). Furthermore, early embryonic death during the first 2 to 5 weeks after fertilization is a source of considerable economic loss to the equine breeding industry, and is particularly prevalent in aged mares (Ball 1988; Morris and Allen 2002). Aged mares are often desirable for breeding stock because they have either competed with distinction or produced earlier offspring that have proven to be gifted athletes. Thus, there are both strong economic and research justifications for examining this particular window of early embryonic development in the horse.

To examine the dynamics of gene expression during early embryo development, one must obtain sufficient quantities of mRNA transcripts for analysis. Real-time quantitative polymerase chain reaction (qPCR) analysis is fast, reliable, and sufficiently sensitive to provide accurate relative quantification of gene expression in small quantities of tissue such as single embryos (Bustin 2002). However, because of its sensitivity qPCR can lead to misinterpretation if proper standardization is not used. Unwanted variation can arise from differences in the amount of tissue used, in the total amount of mRNA in different cells and at different times, and from variation in mRNA extraction, cDNA synthesis by reverse

transcriptase and PCR amplification efficiencies (Vandesompele *et al.* 2002). The use of internally expressed reference genes is one of the most widely adopted methods of compensating for this unwanted variation in qPCR experiments (Vandesompele *et al.* 2002). A good reference gene should be stably expressed across the tissue and treatments of interest, with <2-fold maximum change between samples (de Jonge *et al.* 2007). No universal reference gene exists. Thus a number of studies have demonstrated the need to accurately validate the stability of reference genes in the system under investigation and, once validated, to use multiple reference genes to accurately normalize gene expression data (Thellin *et al.* 1999; Vandesompele *et al.* 2002; Dheda *et al.* 2005).

To date, reliable reference genes have not been described for early pre-implantation embryo development in the horse. Indeed, published papers have mostly used a single non-validated reference gene to normalize gene expression data in early horse embryos. One obstacle to validation is that collecting large numbers of *in vivo* horse embryos is time consuming and expensive, primarily because superovulation regimens are relatively ineffective (Allen 2005). Since embryos are limited and each embryo contains minimal amounts of mRNA (which is also required to investigate the expression of genes of biological interest), we questioned the practicality of testing a large array of historically common reference genes to identify the best 3 or 4 for normalization, as has been reported previously for early pre-implantation embryos of other species (Goossens *et al.* 2005; Kuijk *et al.* 2007; Mamo *et al.* 2007; 2008). Instead, we chose to evaluate a small number of reference genes previously indentified as being stably expressed in developing embryos of other species (Kuijk *et al.* 2007) or across a diverse range of tissues (de Jonge *et al.* 2007). A parallel reference gene study was recently reported (Smits *et al.* 2009) but was restricted to blastocysts derived under various conditions or subjected to cryopreservation (*in vivo* versus *in vitro* versus *in vitro* cryopreserved), after the RNA had been subjected to global amplification; these reference genes may not be suitable for

comparing early equine embryos across different stages of development or for embryos not subjected to prior RNA amplification. Here we demonstrate that using an evidence-based minimalist strategy, we were able to identify stably expressed reference genes suitable as normalization factors for morula to expanded blastocyst stage equine embryos.

Materials and methods

Collection of in vivo embryos

Twenty one embryos were recovered 6.5–7 days after ovulation from 16 Dutch Warmblood mares (aged 4–12 years) inseminated with semen from a single fertile stallion as described previously (Rambags *et al.* 2008). Embryos were recovered by non-surgical uterine lavage using 3 x 1 L pre-warmed (37°C) lactated Ringer's solution (LRS; Baxter, Lessines, Belgium) supplemented with 0.5% fetal calf serum (FCS; Greiner Bio-One, Alphen aan den Rijn, Netherlands). Embryos were then 'washed' through 10 wells of LRS to remove any maternal cells or residual FCS. Embryo diameter was measured using an eye-piece micrometer attached to a SZ60 dissecting microscope (Olympus, Zoeterwoude, Netherlands) and embryos were further classified by developmental stage (morula, early blastocyst or expanded blastocyst; Fig. 1) and quality grade (1–4) as described by Tremoleda *et al.* (2003). Embryos were snap-frozen in liquid nitrogen in 10 µl LRS and stored at –80°C until RNA extraction. All animal procedures were approved by Utrecht University's Animal Experimentation Commission (DEC).

RNA extraction and cDNA synthesis

Total RNA was extracted from whole individual embryos in 600 µl buffer RLT using an AllPrep DNA/RNA/Protein Mini Kit, and subjected to on-column DNase I digestion using an RNase-Free DNase Set (both Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RNA was eluted in a final volume of 35 µl RNase-free water. Since the amount

of RNA recovered from individual embryos in preliminary trials was below the detection limit of a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), all of the RNA from each individual embryo was synthesized into cDNA. +RT samples were synthesized in a 40 µl reaction volume that contained 24 µl embryo RNA, 1x First strand buffer, 5 mM DTT, 0.5 mM dNTPs (Promega, Madison, WI, USA), 600 ng random primers, 40 U RNase In (Promega) and 200 U Superscript III reverse transcriptase (all Invitrogen, Breda, Netherlands unless otherwise stated). To test for genomic DNA (gDNA) contamination, –RT samples were made up in a 20 µl reaction volume that contained 8 µl embryo RNA and the same reagent concentrations but without reverse transcriptase. RNA was added to the reaction mixture after an initial denaturation step of 5 min at 70°C followed by 1 min on ice. Thereafter, the reaction was incubated for 5 min at 25°C, 1 h at 50°C and 5 min at 80°C. The quality and purity of cDNA from each embryo was verified using conventional PCR and generic actin-family primers (that amplify both cDNA and any contaminating gDNA of β-actin, γ1-actin and a hypothetical mRNA product identified in the NCBI horse genome database; Table 1). For the PCR, the total reaction volume was 25 µl containing 1 µl +RT or –RT embryo cDNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.5 µM forward primer, 0.5 µM reverse primer and 0.625 U HotStarTaq DNA polymerase (all Qiagen unless otherwise stated). PCR cycling conditions consisted of 15 min at 95°C followed by 35 cycles of 30 sec at 94°C, 30 sec at primer-specific annealing temperature (see *ACT* Table 1) and 1 min at 72°C; with a final extension of 10 min at 72°C. Products were visualized on 1% agarose gels. If suitable for PCR amplification and free of gDNA contamination, both +RT and –RT embryo cDNA samples were diluted 10-fold and frozen at –20°C in multiple single-reaction aliquots until required for qPCR analysis.

Reference gene selection and primer design

In this study, we tested the expression of four potential reference genes, Phosphoglycerate kinase 1 (*PGK1*), Signal recognition particle 14kDa (*SRP14*), and ribosomal proteins L4 (*RPL4*) and L13A (*RPL13A*). *PGK1* was ranked among the 3 most stably expressed genes in oocytes and throughout pre-implantation embryo development in the pig (Kuijk *et al.* 2007). *SRP14*, *RPL4* and *RPL13A* were ranked among the top 15 most stably expressed genes out of 13,037 unique genes tested across 13,629 different human and 2,543 different mouse gene array samples, derived from a wide variety of different tissues and experimental conditions (de Jonge *et al.* 2007). Most of these top 15 genes were ribosomal and their stability differed according to species (de Jonge *et al.* 2007). Thus, both *RPL4* and *RPL13A* (ranked 2nd and 4th in mouse but 12th and 5th in man respectively) were tested to evaluate which was most stably expressed in horse tissue. In addition, Caudal type homeobox 2 (*CDX2*), was included in the study as a biologically variable 'control' gene. CDX2 is a developmentally regulated transcription factor known to specify trophectoderm during the period of blastocyst formation in the mouse (Strumpf *et al.* 2005). Inclusion of *CDX2* permitted comparison of stability between a developmentally regulated gene and candidate reference genes, and provided a target gene to better assess the effects of normalization. Moreover, this enabled us to characterize the pattern of *CDX2* expression in the developing horse blastocyst since this was not yet known. Equine-specific *CDX2* qPCR primers were obtained from published sequences (de Mestre *et al.* 2009); for the remaining genes, human and mouse mRNA sequences were used to BLAST the NCBI horse genome (NCBI Horse Genome Resources) to identify homologous equine mRNA and gDNA sequences. These were then imported into PerlPrimer v1.1.17 (Marshall 2004) to design intron spanning/intron-exon overlapping mRNA-specific primers suitable for qPCR. The sequence-specificity of primers was confirmed by BLAST analysis against the NCBI horse genome (Table 1).

Primer validation and quantitative PCR

Primers for each gene were optimized and tested for specificity using 10 µl of 100-fold diluted positive control small intestine (for *CDX2*) or testis cDNA. Optimal annealing temperatures (T_A) were determined by temperature gradients that spanned the primer melting temperature (Tm) ±5°C. The optimal T_A for each primer pair gave the highest quantity and purity of PCR product based on the height, and clean single peak, of its melt curve coupled with an early C_q score during qPCR (Table 1). Once optimized, qPCR product from each primer pair was run with a 100 bp DNA ladder (Invitrogen) on a 2% agarose electrophoresis gel and its size was verified using Quantity One v4.3 software (BioRad, Hercules, CA, USA) (Fig. 2). Products were also sequenced using their respective forward and reverse primers in separate sequencing reactions with an ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit and an ABI PRISM 3130xl DNA sequencer (both Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands). Specificity was validated by comparing these sequences with those from the NCBI horse genomic database. Once validated, qPCR products were precipitation-purified and the absolute amount of DNA quantified by Nanodrop spectrophotometer (Nanodrop Technologies), before 5-fold serial dilution in 10 mM Tris-Cl (pH 8.5) to yield 8 different standard solutions ranging from 100 fg to 1.28 ag. Standards were tested in duplicate and the equivalent of 10 µl of 10-, 100- or 500-fold diluted cDNA from each of 3 test embryos was included on the plate to determine if amounts of cDNA from individual embryos could be quantified within the range of the standard curves.

Quantitative PCR was performed to optimize all primers and standards and to run final embryo plates using an iQ5 Real Time PCR Detection System and iQ5 Optical System Software v2.0 (BioRad). The total reaction volume was 25 µl per well containing 1x iQ SYBR Green Supermix (BioRad), 0.5 µM forward primer and 0.5 µM reverse primer (Ocimum Biosolutions, IJsselstein, Netherlands) and, for final embryo plates, one of each of the following samples in separate reactions: (i) 100 fg–6.4 ag of 5-fold diluted standards

(n=7); (ii) 10 µl of 10-fold diluted embryo cDNA (n=21); (iii) 10 µl of 10-fold diluted embryo –RT sample (n=21); or (iv) 10 µl DNase/RNase-free water (Invitrogen) as no-template control. All samples except the –RT, were run in duplicate (technical replicates), with embryos also divided into biological replicates consisting of 5 morulae, 7 early and 9 expanded blastocysts. Both frozen-validated and freshly-prepared standards were included in duplicate on each plate. Due to space limitations, –RT samples were run on a separate plate in a subsequent run on the same day using identical standards. PCR cycling conditions consisted of 4.5 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at optimal T_A and 30 sec at 72°C during which fluorescence was acquired; followed by a melt-curve protocol that consisted of 1 min at 95°C, 1 min at optimal T_A, then 10 sec at optimal T_A increasing to 95°C by 0.5°C per cycle during which a second round of fluorescence was acquired. Baseline and threshold (~100 relative fluorescence units; RFU) values were manually adjusted and samples with non-uniform/failed amplification, primer dimers, or with amplified products in the corresponding –RT sample were excluded from further analysis for all genes.

Reference gene stability and gene expression normalization

geNorm v3.5 software was used to compare the stable expression of each reference gene over the different embryonic development stages as described by Vandesompele *et al.* (2002). Raw, non-normalized data obtained during qPCR detection was exported from the iQ5 software as starting quantities, derived from the standard curve. Relative starting quantities, in which the highest value was set to 1 for each gene, were then calculated and this data was imported into geNorm for analysis. The gene expression stability measure (M) and pair-wise variation (V) for a particular gene compared with all other tested reference genes was calculated as previously described (Vandesompele *et al.* 2002). The optimum number of reference genes to use for normalization was achieved when $V \leq 0.15$; a limit beyond which inclusion of further less-stable reference genes for normalization is considered unwarranted

(Vandesompele *et al.* 2002). The relative expression of all 5 genes among embryos was normalized against the top 3 reference genes using normalization factors obtained by calculating their geometric mean from standard curve derived starting quantities.

Statistical analysis

Data were analyzed using SYSTAT 10.2 (Systat Software, Chicago, IL, USA) and assessed for normal distribution, equal variance between groups, and the presence of outliers (Quinn and Keough 2002). Relationships were tested by pair-wise Pearson's correlation with a post-hoc Bonferroni test. Differences in average gene expression between stages of embryo development were tested by unbalanced ANOVA with a post-hoc pair-wise Bonferroni test. Probabilities (P) $\leq$0.05 were considered to be significant.

Results

Embryo collection and sample quality

Twenty one grade 1–2 embryos were collected; they consisted of 5 morulae, 7 early and 9 expanded blastocysts ranging in diameter from 126–138, 134–196 and 230–680 μm respectively (Fig. 1). Conventional PCR amplification using generic actin primers (Table 1) confirmed that the cDNA from all embryos was suitable for PCR amplification and was free of gDNA contamination, based on the presence or absence of a product in the +RT and –RT samples respectively (data not shown).

Primer validation, quantitative PCR efficiency and relative gene expression

Intron spanning/intron-exon overlapping mRNA-specific primers were designed for *PGK1*, *SRP14*, *RPL4*, *RPL13A* and *CDX2*, and the optimal annealing temperature (T_A) for each primer pair gave rise to a clean single product peak/dissociation temperature during melt curve analysis (Table 1). PCR products for each primer pair were of the expected size when

visualized by agarose gel electrophoresis (Fig. 2) and DNA sequencing confirmed that the products were specific to the target genes of interest (data not shown). Amplification was robust on standards ranging from 100 fg–32 ag (and down to 6.4 ag for *RPL13A* and *CDX2*) and gave amplification efficiencies of 91.4, 98.6, 102.8, 92.8 and 100.9% (R^2=0.985, 0.988, 0.995, 0.995 and 0.993; slope=-3.547, -3.356, -3.258, -3.507, -3.300; y-intercept=29.039, 32.234, 26.371, 27.233, 24.922) for *PGK1*, *SRP14*, *RPL4*, *RPL13A* and *CDX2* respectively. All 5 genes were expressed in all test embryos and the equivalent of 1 µl undiluted or 10-fold diluted embryo cDNA was sufficient to amplify within the working range of the standards for each gene. In the final plates, wells containing primer dimers were excluded from further analysis and expression for all genes was below detectable levels in –RT samples.

All 5 genes were expressed in morula to expanded blastocyst stage equine embryos and there was a strong correlation between the level of expression and embryo size (relative expression vs. embryo diameter R=0.92, 0.88, 0.88, 0.88 and 0.81; P<0.001 for *CDX2*, *RPL4*, *RPL13A*, *SRP14* and *PGK1* respectively; Fig. 3). Pure products were amplified in all embryos; however one duplicate failed to amplify *PGK1* in cDNA from 4 embryos (E24, E31, E16 and E17) and one replicate failed to amplify *CDX2* in cDNA from 2 embryos (E19 and E16). *PGK1* showed the greatest increase in gene expression at the expanded blastocyst stage (368-fold higher) relative to the smallest morula, followed by *CDX2* (332-fold higher). *RPL13A* expression changed the least (117-fold higher) during this period of development. The expression of all genes increased markedly in expanded blastocysts, particularly in those with an embryo diameter >400 µm (E13–E5; Fig. 3).

Reference gene expression stability

A ranking of the stable expression of each reference gene across the different developmental stages, based on the gene expression stability measure (M), is shown in Table 2. *RPL4* was

304 the most stably expressed gene followed by *SRP14* and *RPL13A*, while *CDX2* showed the
305 least stable expression. To test whether *RPL4* and *RPL13A* may be co-regulated, we examined
306 whether removal of one from the analysis significantly affected the stability ranking of the
307 other. Exclusion of either of these ribosomal genes resulted in a single-rank decrease in the
308 apparent stability of the remaining gene, although expression of each was still more stable
309 than *CDX2* (Table 2). These results indicate that the expression patterns of these two genes
310 are so similar that they support each others favourable ranking. Since *RPL4* and *RPL13A* form
311 part of the same ribosomal unit in cells, these results infer that *RPL4* and *RPL13A* may be, at
312 least partially, co-regulated and as such should not be used jointly as normalization factors.
313
314 The pair-wise variation (V) was calculated for the two most stably expressed genes (from
315 each ranking in Table 2) when the next most stable genes were included successively, i.e.
316 V2/3, V3/4 and V4/5 (Fig. 4). We defined the optimal number of genes for normalization as
317 the minimum number sufficient to reduce the pair-wise variation to 0.15, as recommended by
318 Vandesompele *et al.* (2002). With all genes included in the analysis, the pair-wise variation of
319 *RPL4*, *SRP14* and *RPL13A* (V2/3) was already below this threshold (V=0.143), decreased
320 further following the addition of *PGK1* (V3/4), but increased after the addition of the least
321 stably expressed developmentally regulated gene, *CDX2* (V4/5; Fig. 4*a*). Without *RPL4*, the
322 pair-wise variation of *SRP14*, *PGK1* and *RPL13A* (V2/3) did not decrease below the threshold
323 (V=0.167) but did increase following the addition of *CDX2* (V3/4; Fig. 4*b*). However, in the
324 absence of *RPL13A*, the pair-wise variation of *SRP14*, *RPL4* and *PGK1* (V2/3) decreased to
325 the threshold (V=0.151) but increased above this limit after the addition of *CDX2* (V3/4; Fig.
326 4*c*). These results confirm that, in early horse embryos, *RPL4* is more stably expressed than
327 *RPL13A*; as such we recommend the use of *RPL4* in combination with *SRP14* and *PGK1* as
328 suitable reference genes for normalization purposes.
329

330 ***Normalized gene expression***

331 The relative expression levels of all 5 genes for each embryo were normalized against the
332 chosen reference genes (*SRP14*, *RPL4* and *PGK1*; Fig. 5). Given its putative role in early
333 embryonic development, *CDX2* was, not surprisingly, the least stably expressed gene across
334 *in vivo* equine embryos ranging from 126 µm morulae to 680 µm expanded blastocysts. The
335 ratio of the highest expression of this gene (in embryo E25) to the lowest was 9.8-fold, this
336 compared to 1.8 (E5), 1.8 (E4), 2.0 (E27) and 2.5-fold (E24) differences in expression for
337 *SRP14*, *RPL4*, *PGK1* and *RPL13A* respectively (Fig. 5*a*–e). Moreover, the overall pattern of
338 *CDX2* expression across individual embryos was considerably more variable than for any of
339 the potential reference genes.
340
341 Normalized relative expression for all five genes was also examined in the embryos after
342 grouping by developmental stage (morula, early blastocyst and expanded blastocyst; Fig. 6).
343 After grouping, *PGK1* appeared the most stably expressed gene, followed by *SRP14* and
344 *RPL4* with less than 1.1, 1.2 and 1.2-fold difference in expression respectively between the
345 stages (Fig. 6*a*–*c*). The difference in *RPL13A* gene expression was over 1.5-fold and
346 decreased significantly between morula and expanded blastocyst stages, again suggesting that
347 this gene is less suitable for use as a normalization factor across these developmental stages in
348 the horse (P=0.007; Fig. 6*d*). Expression of *CDX2* increased significantly by more than 1.8-
349 fold from the morula to expanded blastocyst stage, consistent with a role in trophectoderm
350 specification (P=0.02; Fig. 6*e*).
351

352 ## Discussion

353 This study identified *SRP14*, *RPL4* and *PGK1* as stably expressed reference genes suitable for
354 use in normalizing expression data from morula to expanded blastocyst stage *in vivo* horse
355 embryos. *RPL13A* was found to be less suitable due to its probable co-regulation with *RPL4*

and less stable expression which changed significantly during development. *CDX2* expression increased during embryo development, consistent with a role in the formation of trophectoderm, as described for mouse embryos. The pattern of expression described here for equine embryos, suggests that the function of *CDX2* is conserved between mouse and horse.

During this study, we were able to demonstrate that an evidence-based minimalist approach to the selection of potential reference genes can reduce the number that need to be tested to identify a pool suitable for normalization purposes; this is particularly relevant for early-stage embryos or other biological materials where minimal amounts of mRNA are available. Two of the 3 final reference genes (*SRP14* and *RPL4*) tested in this study were chosen based on their highly stable expression in more than 2,500 different mouse and 13,600 human gene array samples (ranked 6^{th} and 2^{nd} in mouse, and 7^{th} and 12^{th} in man respectively out of 13,037 genes tested; de Jonge *et al.* 2007). *RPL4* also ranked among the 2 most stably expressed reference genes tested across 17 different porcine tissues, and in regenerating mouse liver (Nygard *et al.* 2007; Takagi *et al.* 2008); while *SRP14* ranked among the 2 most stably expressed genes in human myocardium (Pilbrow *et al.* 2008). Since care should also be taken to select an array of genes that is relevant to the samples under investigation, *PGK1* was also selected. Previous work in our laboratory identified *PGK1* as a very stably expressed gene during porcine pre-implantation embryo development (Kuijk *et al.* 2007); *PGK1* also ranked among the 3 most stably expressed genes in rat oligodendrocytes and differentiating mouse and human embryonic stem cells (Willems *et al.* 2006; Nelissen *et al.* 2010). Moreover, the stability ranking and pair-wise variation of the best 3 reference genes identified in the current study were lower than the 4 best reference genes advocated in the equine expanded blastocyst study reported by Smits *et al.* (2009), suggesting that they may be valuable additions to the pool of potential reference genes for studies on early equine embryo development.

RPL4 and RPL13A are both ribosomal proteins that form part of the large 60S subunit which is responsible for translational elongation (Dresios *et al.* 2006). Although evidence from archaeal homologues suggests that they do not directly interact (Ban *et al.* 2000), and although each has been reported to have separate and distinct extra-ribosomal functions in prokaryotes and man (Warner and McIntosh 2009), it is likely these genes are co-regulated. Functionally co-regulated genes can confound geNorm analysis because their pair-wise variation will be smaller across experimental treatments than unrelated genes, leading to an overestimate of expression stability. *RPL4* and *RPL13A* were both included in the current study to determine which was more stably expressed in horse embryos, since they have been shown to exhibit species-specific differences (ranked 2nd and 4th in mouse but 12th and 5th in man respectively; de Jonge *et al.* 2007). Exclusion of one of these two ribosomal genes from geNorm analysis negatively affected the stability ranking of the other (Table 2), supporting the hypothesis that these genes are co-regulated and as such should not be used jointly as normalization factors. In this respect, *RPL13A* was discounted as a reference gene because its expression was less stable than *RPL4*, and not sufficient to reduce the pair-wise variation of the 3 best reference genes below the recommended threshold (Table 2 and Fig. 4*b*). After normalization, *RPL13A* gene expression was shown to decrease significantly during morula to expanded blastocyst development (Fig. 6*d*) and, although the magnitude of this change was small (just over 1.5-fold), it does suggest that *RPL13A* is not stably expressed under these conditions, and its use as a reference gene is thus not recommended.

CDX2 was included as a 'developmentally regulated' control in this study because it is known to be highly regulated during blastocyst formation in the mouse (Strumpf *et al.* 2005). As expected, *CDX2* had the lowest stability ranking and greatest negative effect on increasing pair-wise variation among the tested genes (Table 2 and Fig. 4). In addition, normalized *CDX2* expression was more variable across individual embryos with a nearly 10-fold

difference between the highest and lowest expression (Fig. 5*e*). Interestingly, when embryos were grouped by stage of development, *CDX2* expression increased significantly nearly 2-fold from the morula to the expanded blastocyst stage (Fig. 6*e*). This pattern is consistent with an ever-increasing proportion of cells with a trophectodermal phenotype, suggesting that, as in the mouse (Strumpf *et al.* 2005), *CDX2* plays a role in trophectoderm specification in the horse.

Examination of gene expression in single embryos in this study permitted us to observe biological variation often masked when pooled embryos are compared. The strong correlation ($R>0.8$) observed in single embryos between relative gene expression and embryo diameter for all 5 genes, coincides with a rapid increase in cell number as horse embryos develop from a morula into an expanded blastocyst (from 160 to over 2,900) (Rambags *et al.* 2005). Clearly, horse embryos, even those of the same developmental stage, differ greatly from one another in size and cell number. However, good normalization should be able to compensate for these changes, and the combination of *SRP14*, *RPL4* and *PGK1* appears to do this effectively (compare Fig. 3 and 5). Although this single sample approach has been advocated previously (Jolly *et al.* 2005), it has not been widely used for pre-implantation embryos (Mamo *et al.* 2007; 2008).

In summary, we have validated *SRP14*, *RPL4* and *PGK1* as a suitable pool of reference genes for normalizing gene expression data for morula to expanded blastocyst stage *in vivo* equine embryos; this should assist in studies to examine expression of genes potentially involved in normal or compromised development in this species. Due to its probable co-regulation with *RPL4* and less stable expression that changes during development, *RPL13A* is not advocated as a reference gene in this system.

Author contributions

DP designed and performed all experiments, analysed the data and was the primary author of the manuscript. EK assisted with experimental design and technical support, BR assisted with experimental design, data analysis and provided laboratory resources. TS supervised the study, assisted with experimental design and provided veterinary support for animal procedures. All authors examined the data, read and approved the final manuscript.

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Figure Legends

Fig. 1. Representative pictures of *in vivo* produced equine embryos used in this study. (*a*) morula (embryo E4) with a thick zona pellucida; (*b*) early blastocyst (embryo E9) with a thin zona pellucida, ring of developing trophectoderm (arrow) and signs of cavitation; and (*c*) expanded blastocyst (embryo E13) with a thin capsule (arrowhead), a large blastocoel cavity and a clearly identifiable inner cell mass (arrow). zp, zona pellucida; c, cavitation; scale bar=100 µm.

Fig. 2. PCR product size for candidate reference genes. Products separated on a 2% agarose gel in parallel with a 100 bp DNA ladder (L), were of the expected sizes (*PGK1* 260 bp; *RPL4* 203 bp; *RPL13A* 198 bp; *SRP14* 100 bp; *CDX2* 136 bp). Numbers shown indicate DNA fragment size (bp) of the ladder.

Fig. 3. Relative gene expression for individual equine embryos. Embryos arranged in order of increasing embryo diameter (µm) and developmental stage (embryos E24–E4, morulae; embryos E17–E21, early blastocysts; embryos E30–E5, expanded blastocysts). The mean and range (error bars) of technical duplicates are plotted. Gene expression in the smallest embryo was taken as the reference to calculate relative amounts as development progressed.

Fig. 4. Determination of the optimal number of reference genes for normalization. Pair-wise variation (V) between two sequential normalization factors containing an increasing number of less stable reference genes analysed (*a*) for all genes; (*b*) without *RPL4*; and (*c*) without *RPL13A*. An arbitrary cut-off of V≤0.15 (dashed line) was used as a limit below which inclusion of further reference genes for normalization is unwarranted (Vandesompele *et al.* 2002).

Fig. 5. Normalized gene expression in individual equine embryos. Embryos arranged in order of increasing embryo diameter and developmental stage as outlined in Fig. 3. Data were normalized against the 3 best reference genes (*SRP14*, *RPL4* and *PGK1*) and plotted as the mean and range (error bars) of technical duplicates. The embryo with the lowest expression for each gene was taken as the reference to calculate relative amounts for all embryos.

Fig. 6. Normalized gene expression in equine embryos grouped by developmental stage. Stages arranged in order of advancing development. Data were normalized against the 3 best reference genes (*SRP14*, *RPL4* and *PGK1*) and plotted as mean ±SEM of biological replicates. The developmental stage with the lowest expression for each gene was taken as the reference to calculate relative amounts for all stages. Values not sharing the same letter differ significantly ($P \leq 0.05$).

Tables

Table 1. Primer details for candidate reference genes used in quantitative PCR

Symbol	Gene name	GenBank accession number	Primer location	Sequence	Product size (bp)*	T_A (°C)	C_q range
ACT†	actin (β, γ1, hypothetical product)	NM_001081838 XM_001488883 XM_001487824	exon 2–3 exon 1–2 exon 1	forward 5'-GGCACCACACCTTCTACAAC-3' reverse 5'-CGACATAGCAGAGCTTCTCC-3'	402 (850) 402 (680) 402 (402)	67.0–57.0‡	not applicable
PGK1	phosphoglycerate kinase 1	XM_001502668	exon 9–10/11	forward 5'-CAAGAAGTATGCTGAGGCTG-3' reverse 5'-AGGACTTTACCTTCCAGGAG-3'	260	57.0	20.6–34.8
SRP14	signal recognition particle 14kDa	XM_001503583	exon 2/3–3	forward 5'-CTGAAGAAGTATGACGGTCG-3' reverse 5'-CCATCAGTAGCTCTCAACAG-3'	100	55.0	23.6–37.1
RPL4	ribosomal protein L4	XM_001497094	exon 6/7–8	forward 5'-CATCCCTGGAATTACTCTGC-3' reverse 5'-CGGCTAAGGTCTGTATTGAG-3'	203	61.5	18.3–31.9
RPL13A	ribosomal protein L13A	XM_001491876	exon 6/7–8	forward 5'-CTACACGAAAGTTTGCCTACC-3' reverse 5'-TTGAGGACCTCTGTGTATCTG-3'	198	61.5	19.9–34.3
CDX2	caudal type homeobox 2	XM_001915508	exon 3–4	forward 5'-CAGTCGGTACATCACCATCC-3' reverse 5'-GCTGCTGCTGCAACTTCTTC-3'	136	61.4	17.9–33.0

† used to check quality and gDNA contamination of cDNA only

* numbers in parentheses represent gDNA amplicons

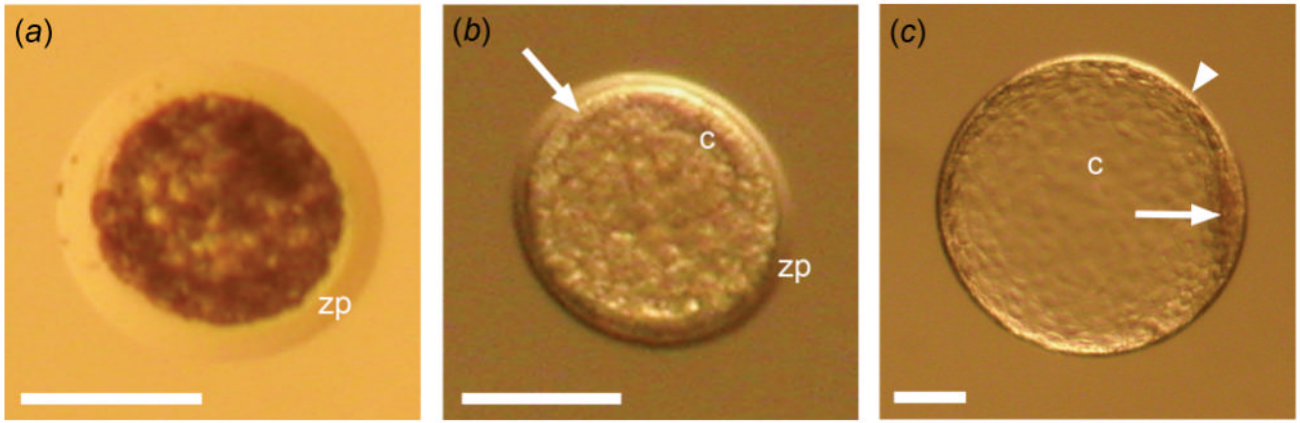
‡ touchdown protocol decreasing from 67 to 57 °C (1 °C per cycle over the first 10 cycles)

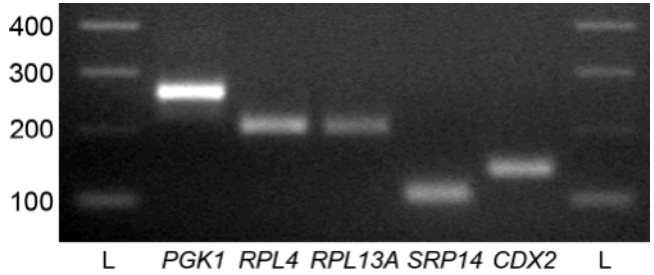
Table 2. Ranking of reference genes by gene expression stability measure (M)

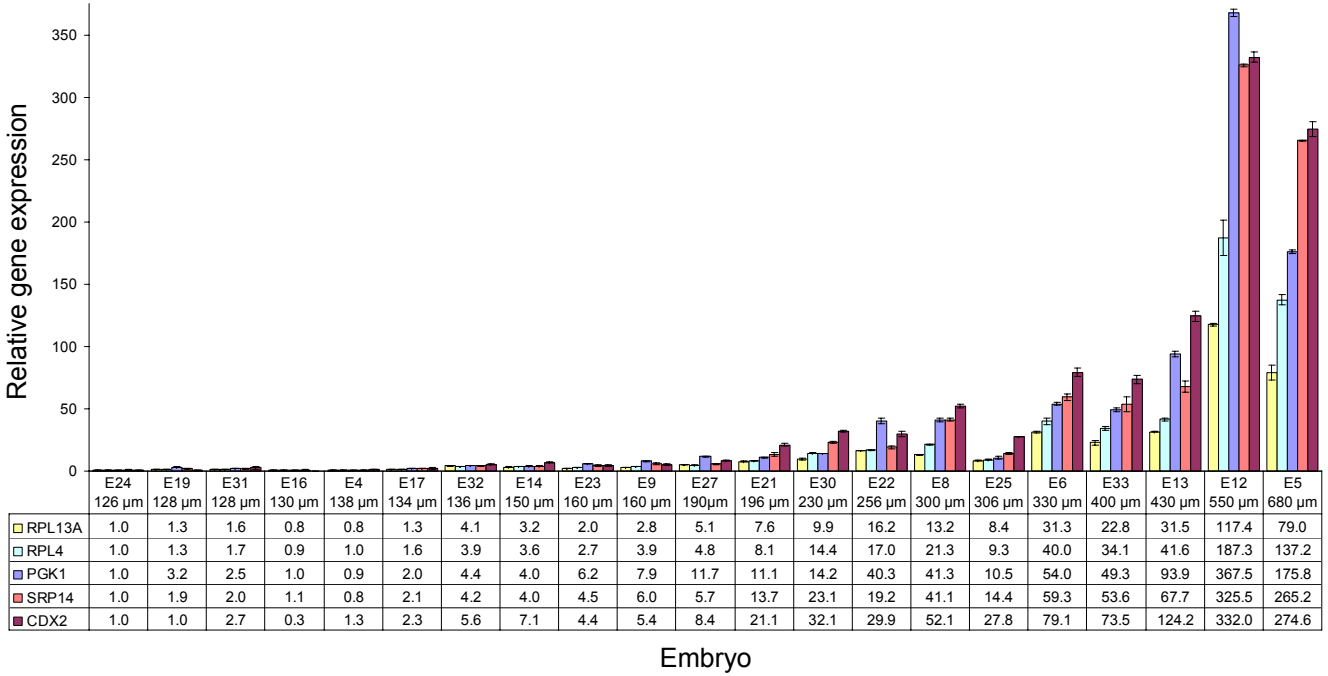
Ranking†	Gene (M value)		
	All genes included	Minus *RPL4*	Minus *RPL13A*
1	*RPL4* (0.463)	*SRP14* (0.567)	*SRP14* (0.497)
2	*SRP14* (0.508)	*PGK1* (0.631)	***RPL4* (0.528)**
3	*RPL13A* (0.566)	***RPL13A* (0.666)**	*PGK1* (0.606)
4	*PGK1* (0.596)	*CDX2* (0.809)	*CDX2* (0.766)
5	*CDX2* (0.797)	-	-

† less stably expressed genes have higher M values

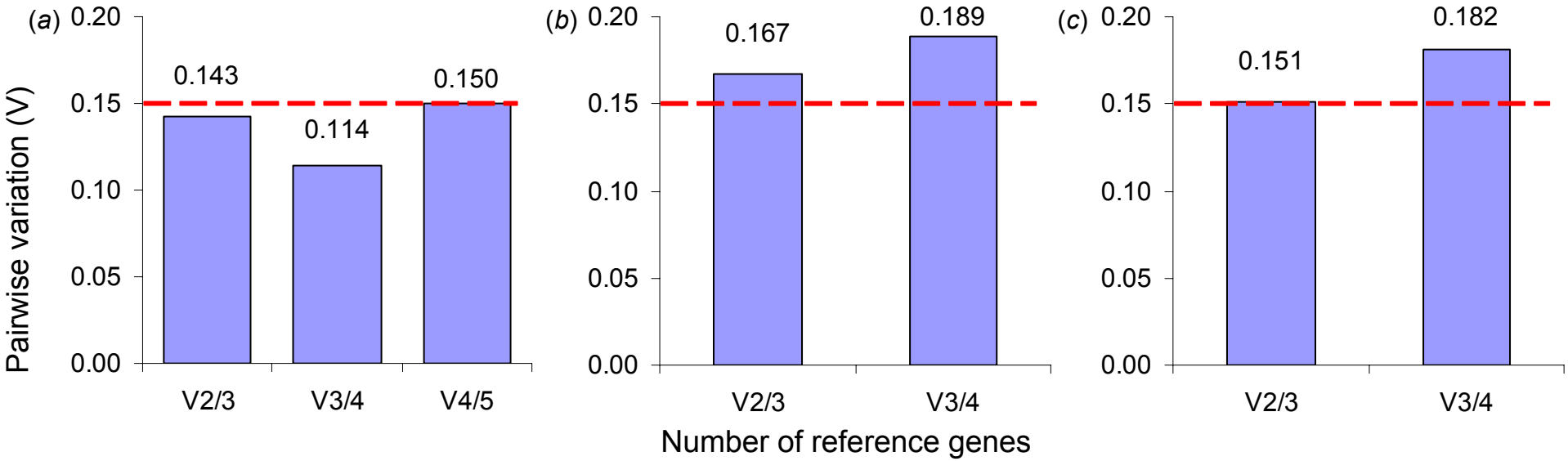
bold=gene negatively affected by exclusion


(a)
zp
(b)
c
zp
(c)
c


400
300
200
100
L
PGK1
RPL4
RPL13A
SRP14
CDX2
L



	E24 126 µm	E19 128 µm	E31 128 µm	E16 130 µm	E4 138 µm	E17 134 µm	E32 136 µm	E14 150 µm	E23 160 µm	E9 160 µm	E27 190µm	E21 196 µm	E30 230 µm	E22 256 µm	E8 300 µm	E25 306 µm	E6 330 µm	E33 400 µm	E13 430 µm	E12 550 µm	E5 680 µm
RPL13A	1.0	1.3	1.6	0.8	0.8	1.3	4.1	3.2	2.0	2.8	5.1	7.6	9.9	16.2	13.2	8.4	31.3	22.8	31.5	117.4	79.0
RPL4	1.0	1.3	1.7	0.9	1.0	1.6	3.9	3.6	2.7	3.9	4.8	8.1	14.4	17.0	21.3	9.3	40.0	34.1	41.6	187.3	137.2
PGK1	1.0	3.2	2.5	1.0	0.9	2.0	4.4	4.0	6.2	7.9	11.7	11.1	14.2	40.3	41.3	10.5	54.0	49.3	93.9	367.5	175.8
SRP14	1.0	1.9	2.0	1.1	0.8	2.1	4.2	4.0	4.5	6.0	5.7	13.7	23.1	19.2	41.1	14.4	59.3	53.6	67.7	325.5	265.2
CDX2	1.0	1.0	2.7	0.3	1.3	2.3	5.6	7.1	4.4	5.4	8.4	21.1	32.1	29.9	52.1	27.8	79.1	73.5	124.2	332.0	274.6


(a)
(b)
(c)
Pairwise variation (V)
0.20
0.15
0.10
0.05
0.00
0.143
0.114
0.150
0.167
0.189
0.151
0.182
V2/3
V3/4
V4/5
Number of reference genes