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Identification of stable endogenous reference genes for real-time PCR in the human fetal gonad using an external standard technique

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Running title: Identification of stable endogenous reference genes

Abstract

Measurement of tissue mRNA transcript levels is critically dependent upon the normalization strategy used. For real-time PCR (RT-PCR) this commonly depends upon identification of stable (non-variable) endogenous reference genes with housekeeping genes (HKG) the most commonly used. In this report we describe the use of an external mRNA standard to identify stable HKGs in the human fetal gonad. Total RNA was extracted from second trimester human fetal gonads and a standard amount of *luciferase* mRNA was added at the start of the extraction process. Levels of *luciferase* were then measured relative to each of seven HKGs (*SDHA*, *TBP*, *B2M*, *PMM1*, *SFRS4*, *HMB*S and *UBC*) by RT-PCR. When normalised to tissue weight, HKG expression was constant across fetal ages. Measurement of overall variation in transcript expression showed that *PMM1* was the most stable HKG in the ovary while *B2M* was most stable in the testis. Re-analysis of the data using GeNorm and Normfinder algorithms showed that two of the top three most stable HKGs were the same using all three methods. This study describes a method for identification of endogenous, stable reference genes for RT-PCR studies of transcript expression levels which is objective and not dependent on prior assumptions of HKG expression. This technique is likely to be applicable to most tissues and, in this case, identifies suitable HKGs for studies into human gonadal development.

Key words: testis, ovary, real-time PCR, housekeeping genes, reference genes

Introduction

The relevance and meaning of much biochemical data is dependent upon the normalization strategy and this is particularly the case for mRNA quantitation using real-time PCR. Most commonly, normalization is to an endogenous reference gene and the overwhelming majority of the reference genes used for normalization are housekeeping genes (HKG). These HKG carry out essential cell maintenance functions and are used as reference genes on the assumption that they will show stable expression – ie transcript levels will not show developmental-, treatment- or pathological-regulation. It has been shown, however, that many of the most commonly used HKGs do not fulfill these criteria (Huggett *et al*., 2005) and, indeed, a HKG which is universally stable across all tissues almost certainly does not exist. To identify the optimal normalisation strategy for a particular tissue or cell type it is necessary, therefore, to examine the stability of a number of reference genes using algorithms such as GeNorm and Normfinder (Vandesompele *et al*., 2002; Andersen *et al*., 2004). These algorithms make certain assumptions, however, that may not always be valid. For example, the most commonly used algorithm, GeNorm, is based on the hypothesis that the most stable HKGs will show the least variation relative to each other (Vandesompele *et al*., 2002). Low relative variation between HKGs may arise, however, for reasons such as co-regulation and may not necessarily indicate high tissue stability (Andersen *et al*., 2004; Ellefsen *et al*., 2008). An alternative strategy for normalization of real-time PCR data is to use an external "foreign" mRNA as standard, added at the stage of mRNA extraction (Baker and O'Shaughnessy 2001b; Smith *et al*., 2003; Johnson *et al*., 2005; Ellefsen *et al*., 2008). This provides an objective measure of tissue transcript levels relative to the mass of the starting tissue without assumptions of reference gene stability. This technique is not always practical, however, if the extracted RNA is archival, tissue mass or cell number

is unknown or there has been partial RNA degradation before extraction. Nevertheless, the external standard method can be used with a characterized tissue set to assess the relative stability of potential HKGs in that tissue (O'Shaughnessy *et al*., 2002; Ellefsen *et al*., 2008). In this report we have expanded the use of the external standard to rate the stability of HKGs in human fetal gonads without assumptions of co-variability or reliance on model-based estimates of expression variance (Vandesompele *et al*., 2002; Andersen *et al*., 2004). This approach has general applicability to other tissues and systems and can be used to identify the most appropriate HKG for studies in which an endogenous reference gene is necessary.

Materials and Methods

Tissues

The collection of fetal material was approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). For a detailed explanation see O'Shaughnessy *et al*. (2007). Women seeking elective, medical, terminations of pregnancy were recruited with full written, informed, consent by nurses working independently at Aberdeen Pregnancy Counseling Service. There was no change in patient treatment or care and women were able to withdraw from the study at any point. Only normally-progressing pregnancies from women over 16 years of age and between 11-21 weeks of gestation were collected. Fetuses were transported to the laboratory within 30 minutes of delivery. The gonads were weighed, snap-frozen in liquid nitrogen and stored at -85°C. For this study gonads were collected from 22 male and 22 female fetuses.

RNA extraction and reverse transcription

To extract RNA whole testes or ovaries were homogenized in Trizol (Invitrogen Ltd, Paisley, UK) according to the manufacturer's protocol. Immediately before homogenization an aliquot of *luciferase* mRNA (5ng, Promega UK, Southampton, UK) was added to the Trizol and extracted along with the endogenous RNA. The extracted RNA was stored in liquid N_2. Nucleic acid concentration and quality was determined using a NanoDrop (Fisher Scientific UK Ltd, Loughborough, Leicestershire) with expected 260/280 ratios between 1.9 and 2.1. First strand cDNA was synthesized from 2µg of total RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III, Life Technologies, Paisley, UK) as previously described (Rajapaksha *et al*., 1997).

Real-time PCR

The SYBR green method was used for real-time PCR in a 96-well plate format using a Stratagene MX3000 cycler. Reactions contained 5 µl 2 x SYBR mastermix (Agilent Technologies, Wokingham, UK), primer (100 nM) and template in a total volume of 10µl. At the end of the amplification phase a melting curve analysis was carried out on the products formed. No template controls were run on each plate as appropriate for the primer sets under study. All primers were designed by Primer Express 2.0 (Applied Biosystems, Warrington, UK) with parameters set for amplicon length to be between 160 and 200bp with a Tm less than 85^{o}C and primer Tms to be between 64 and 66^{o}C (Baker and O'Shaughnessy, 2001). All primers were designed so that the amplicon crossed an intron and genomic DNA would not amplify. This was tested for each primer pair using samples containing genomic DNA and with a positive control designed to amplify genomic DNA. The primers used are described in Table 1. The amplification efficiencies

of the primer pairs used were between 94 and 102% as determined using sample dilution curves.

Data analysis

The threshold cycle (Cq) value for each sample was determined with each primer pair in duplicate. Levels of *luciferase* mRNA in each sample were then expressed relative to each HKG using the ΔCq method ($2^{Cq(HKG)-Cq(luciferase)}$). The coefficient of variation (CV) for each HKG was determined as the standard deviation/mean of all samples. To determine whether there were age-dependent changes in HKG expression the data was analysed by linear regression using Prism (GraphPad, La Jolla, USA) with $P<0.05$ considered significant. Data was also analysed using GeNorm v3.5 and NormFinder algorithms as previously described (Vandesompele *et al*., 2002; Andersen *et al*., 2004).

Results

The HKGs that were assessed in this study were TATA box binding protein (*TBP*), phosphomannomutase 1 (*PMM1*), hydroxymethylbilane synthase (*HMBS*), ubiquitin C (*UBC*), beta-2-microglobulin (*B2M*), succinate dehydrogenase (*SDHA*) and splicing factor, arginine/serine-rich 4 (*SFRS4*). Levels of *luciferase* mRNA in extracted gonadal tissue was determined relative to each HKG by real-time PCR and the untransformed data, expressed as a function of age, are shown in Supplementary Figs 1 and 2. The weight of both testes and ovaries increased throughout the second trimester (Supp Figs 1 and 2) and, since a standard amount of *luciferase* mRNA had been added to each gonad at the extraction phase, the amount of *luciferase*, relative to each HKG, decreased significantly with age. The only exceptions to this were *UBC* in the testis and *HMBS* in the ovary, relative to which *luciferase* levels did not change significantly with age. To

correct for tissue mass the data shown in the Supplementary Figures was multiplied by the weight of each individual gonad. Following this correction, and with one exception, the amount of *luciferase* measured in each gonad did not change significantly with age, relative to each HKG, throughout the second trimester (Figs 1 and 2). The one exception was when *luciferase* was expressed relative to HMBS in the ovary. In this case there was a significant increase in measured *luciferase* with age (Fig 2) which is likely to be due to an actual decrease in *HMBS* with age. These data show that, with the exception of *HMBS* in the ovary, the levels of each HKG were constant throughout the second trimester, relative to the mass of the tissue.

To determine which HKG showed the most stability between individual gonads the coefficient of variation (CV) for each HKG was determined as the standard deviation/mean using data normalised to gonad weight (Figs 1 and 2). The HKGs with the lowest CVs (ie most stable) were *B2M* in the testis and *PMM1* in the ovary (Figs 1-3). To determine whether the overall CV was reduced by combining data from different HKGs, the results from each HKG were combined in the order of stability (ie most stable HKG, combined with second most stable etc). Results in Fig 3 show that combining the most stable HKGs slightly reduced the CV in the testis but had no effect in the ovary. For both ovary and testis, addition of the least stable HKGs led to an increase in CV.

An overall analysis of HKG transcript levels in all samples was carried out to determine whether any samples had markedly reduced levels of all HKGs, which would be an indication of mRNA degradation prior to tissue collection and storage. None of the samples showed apparently high levels of *luciferase* across all HKGs (which would mean that HKG levels were low) (Supplementary Fig 3)

To compare these results with the other methods of identifying stable HKGs the data were analysed using GeNorm v3.5 and NormFinder algorithms as previously described (Vandesompele *et al*., 2002; Andersen *et al*., 2004) (Fig 4). The order of HKG stability, in both testis and ovary, was very similar using GeNorm and NormFinder (Table 2). The most stable two HKGs identified by GeNorm and NormFinder in both ovary and testis also ranked highly using the external standard method described here with *PMM1* ranked the most stable HKG in the ovary by all three methods. It is worth noting, however, that *B2M* was ranked as the most stable HKG in the testis using the external standard method but was ranked second or third *least* stable using GeNorm or NormFinder (Table 2).

Discussion

Appropriate normalization of real-time PCR data is essential to generate meaningful results. A number of groups have described strategies based on use of external standards (Baker and O'Shaughnessy, 2001; Ellefsen *et al*., 2008; Bower *et al*., 2007; Johnson *et al*., 2005; Smith *et al*., 2003) but the method appears to have been adopted for routine use by only a very small minority. This is surprising since it overcomes many of the inherent problems associated with use of endogenous genes. There are a number of situations in which it is not practical to use an external standard, as outlined in the introduction, but the most likely reason that HKGs remain so popular is that historically they have been routinely used in studies of transcript levels (eg for Northern blots) and there may be an inherent inertia to the adoption of other methods. Whatever the reason, since reference to a HKG is likely to remain the normalization strategy in the overwhelming majority of real-time studies it is essential that the most appropriate HKG is identified for each tissue, age and pathology of interest.

The external standard method for assessing HKG stability is straightforward and generally applicable although it is not assumption-free. Firstly, it is implicit that the exogenous mRNA will be extracted with either the same efficiency as the endogenous mRNA or, at least, at a constant ratio. In soft tissues such as the human fetal gonad this is likely to be valid as they can be homogenized quickly and completely with rapid denaturation of proteins and mixing of exogenous and endogenous RNA. It is possible that variation in extraction efficiency between endogenous and exogenous RNA may contribute to overall variance but if it was a major component then it would be expected to affect all HKGs in any one sample in the same way. There is little evidence from Supplementary Fig 3 to suggest a consistent pattern within samples of high deviation either above or below the mean. The second assumption is that there is a linear relationship between tissue mass and total mRNA. This relationship is likely to hold true unless changes in tissue mass are due to accumulation or loss of fluid or extracellular matrix composition. In a tissue like the developing gonad increased tissue mass is due largely to cell proliferation and is likely to reflect changes in mRNA content. Overall, therefore, for soft tissues like the gonads the assumptions are probably valid but they may need to be considered more carefully for some other tissues or pathologies.

Recognition that the traditionally used HKGs such as β-actin and GAPDH show differential expression (Huggett *et al*., 2005) led to the development of techniques such as GeNorm and NormFinder which objectively compare HKG stability in a given tissue. Any technique which attempts to identify stable HKG from endogenous expression levels alone is caught in the conundrum that to identify the most stable HKG you have to already know which HKG is most stable. To circumvent this, assumptions are made about the likely co-variance patterns of stable HKGs or model-based strategies are used to estimate expression variability (Vandesompele *et al*., 2002; Andersen *et al*., 2004).

From our data, these assumptions appear to be largely valid for the developing human gonad in that two of the top three most stable transcripts were the same in all three methods. Interestingly, however, in both ovary and testis *B2M* was ranked among the least stable HKGs by GeNorm and Normfinder but was the most stable in the testis and the second most stable in the ovary as assessed using the external standard method. This indicates that some apparently stable HKGs may not fulfill the criteria of GeNorm and Normfinder and may be erroneously excluded from normalization strategies. Erroneous exclusion of a stable HKG will reduce the normalization power of an experiment but is far less damaging than including an unstable gene in the normalization algorithm.

In conclusion, this study describes a straightforward method for objective identification of stable reference genes which does not rely upon variability models or assumptions of co-variance. Further data from a number of tissues are needed but results from the developing human gonad also confirm that GeNorm and Normfinder will identify stable HKGs although they may also erroneously exclude suitable candidates. The stable HKGs identified in this study will be of use in future studies of transcript levels in the developing human gonad.

Authors' role

POS conceived the study, analysed data and drafted the manuscript. AM carried out reverse transcription and real-time PCR studies. PF provided samples, participated in the design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Table 1 Primers

Gene	Genbank	Primers
TATA box binding protein (TBP)	nm_003194	aggaaaaaattgaatagtgagacgagttcca tggactaaagatagggattccgggagt
Phosphomannomutase 1 (PMM1)	nm_002676	aacatctcgcccatcggcc tcaaagctgatcatgcctcctcg
Hydroxymethylbilane synthase (HMBS)	nm_000190	gctggcacaaccgggtgg ttcagcgatgcagcgaagca
Ubiquitin C (UBC)	nm_021009	ggcggtgaacgccgatgatt agggtgatggtcttaccagtcagagtctt
Beta-2-microglobulin (B2M)	nm_004048	tcgctccgtggccttagctg caatgtcggatggatgaaacccag
Succinate dehydrogenase (SDHA)	nm_004168	acgtcacgaaggagccgatcc atgtaccgaggcacaggcgg
Splicing factor, arginine/serine-rich 4 (SFRS4)	nm_005626	cgggaagatcctggaggtgga cgtaactgccatctcgccgtg

Table 2 Reference gene stability

A) testis

Stability Ranking	Method: Normfinder	GeNorm	External standard
1.	*SDHA*	*SDHA/TBP**	*B2M*
2.	*TBP*	*PMM1*	*TBP*
3.	*PMM1*	*SFRS4*	*SDHA*
4.	*SFRS4*	*B2M*	*SFRS4*
5.	*HMBS*	*HMBS*	*PMM1*
6.	*B2M*	*UBC*	*HMBS*
7.	*UBC*		*UBC*

B) ovary

Stability Ranking	Method: Normfinder	GeNorm	External standard
1.	*PMM1*	*PMM1/TBP**	*PMM1*
2.	*TBP*	*SFRS4*	*B2M*
3.	*SFRS4*	*SDHA*	*TBP*
4.	*SDHA*	*B2M*	*HMBS*
5.	*B2M*	*UBC*	*SDHA*
6.	*UBC*	*HMBS*	*SFRS4*
7.	*HMBS*		*UBC*

*GeNorm does not differentiate between the two most stable genes.

Legends

Figure 1

Luciferase mRNA relative to 7 different HKGs in the fetal human testis. Data shows *luciferase* mRNA measured relative to each HKG in testes of different ages. The data has been corrected for tissue mass by dividing each data point by the relevant testis weight. . Each point represents an individual testis and the significance of age-dependent changes was determined by linear regression. The dotted line is the mean value and the solid line is the regression line. The regression slope was not significantly different to zero for any HKG. The range of the Y-axis has been set at 6 times the mean value.

Figure 2

Luciferase mRNA relative to 7 different HKGs in the fetal human ovary. Data shows *luciferase* mRNA measured relative to each HKG in ovaries of different ages. The data has been corrected for tissue mass by dividing each data point by the relevant ovarian weight. . Each point represents an individual ovary and the significance of age-dependent changes was determined by linear regression. The dotted line is the mean value and the solid line is the regression line. The regression slope was not significantly different to zero for any HKG apart from HMBS. The range of the Y-axis has been set at 6 times the mean value.

Figure 3

Coefficient of variation (CV) for each HKG in the testis and the ovary (A) and effect of combining data from different HKGs on the overall CV (B). Data in A is taken from Figs

1 and 2. To combine data in (B) the geometric mean of different HKGs for each sample was calculated and used to determine the CV.

Figure 4

HKG stability as assessed using GeNorm and Normfinder algorithms in ovary (A) and testis (B). Data plotted in supplementary Figs 1 and 2 were subjected to GeNorm and Normfinder analysis.

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Figure 1

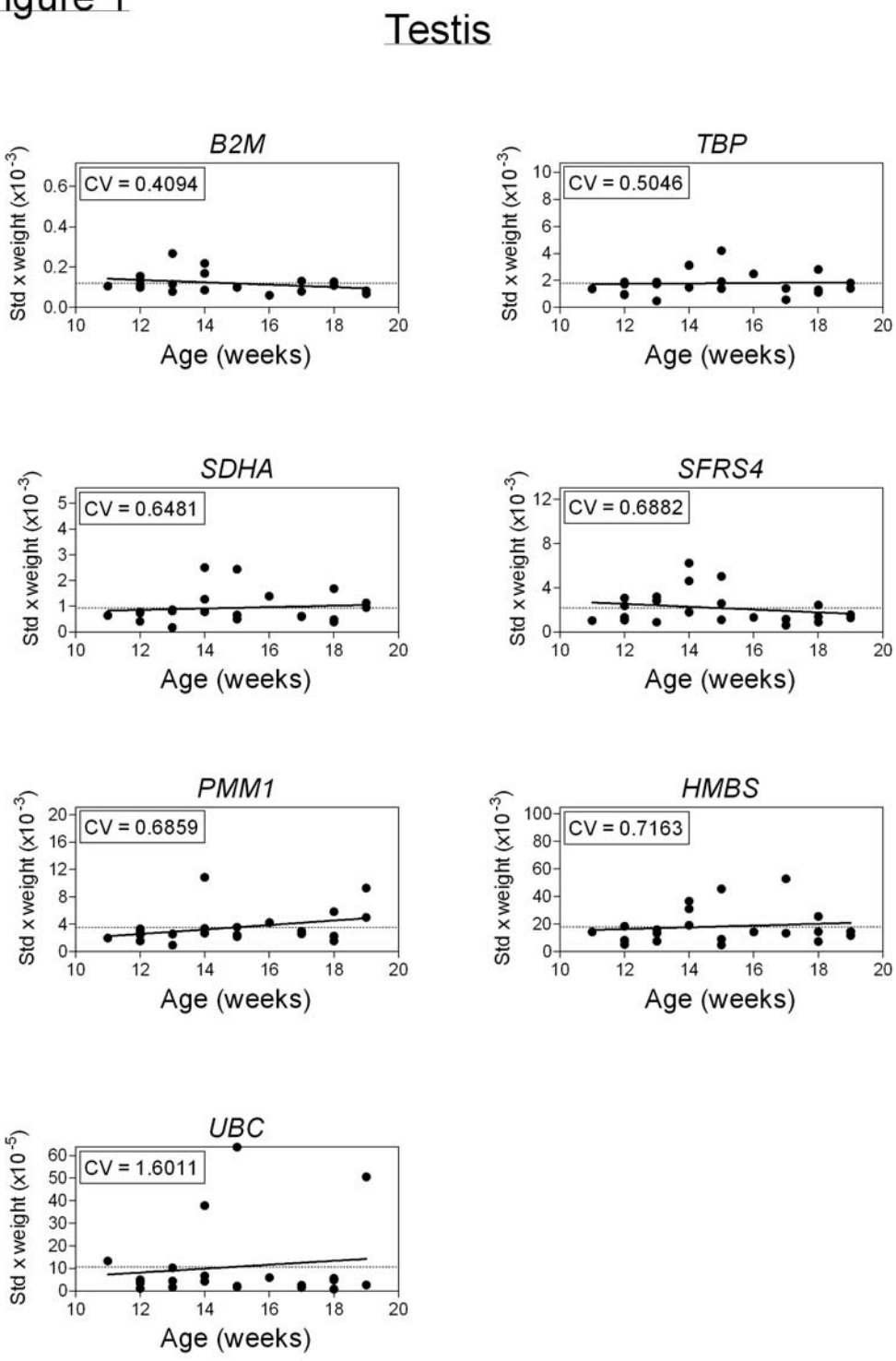

Testis
B2M
CV = 0.4094
Std x weight (x10-3)
Age (weeks)
TBP
CV = 0.5046
SDHA
CV = 0.6481
SFRS4
CV = 0.6882
PMM1
CV = 0.6859
HMBS
CV = 0.7163
UBC
CV = 1.6011
Std x weight (x10-5)

Figure 2

Ovary

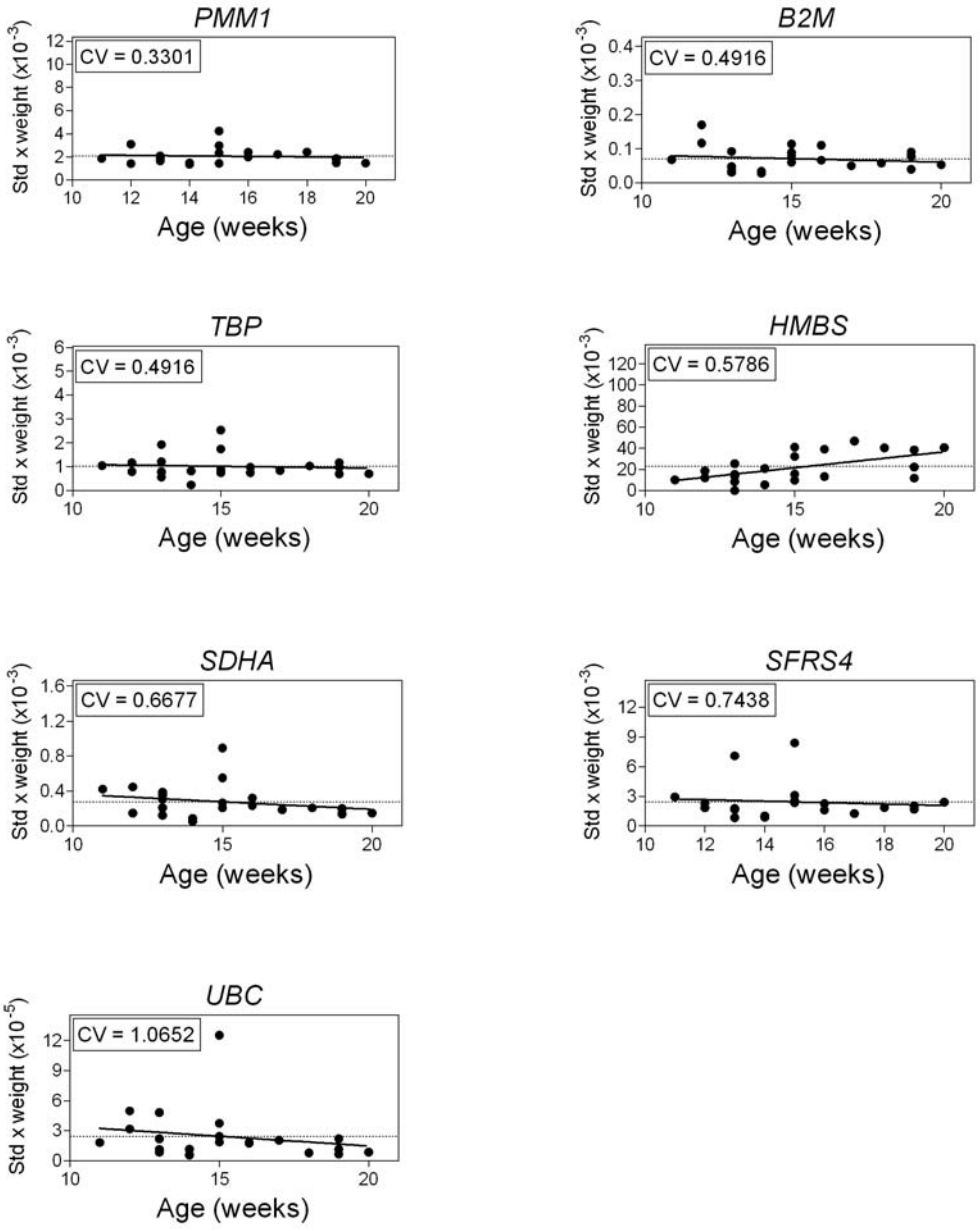
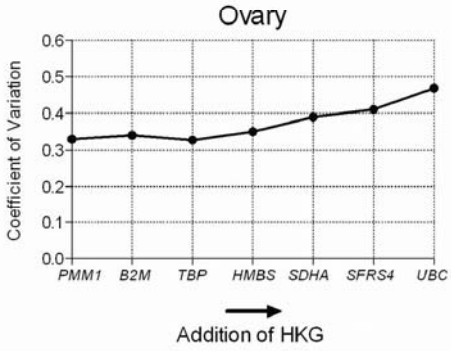


Figure 3

A) CV for each HKG

	CV	
HKG	Ovary	Testis
B2M	0.492	0.409
TBP	0.492	0.505
SDHA	0.668	0.648
PMM1	0.330	0.686
SFRS4	0.744	0.688
HMBS	0.579	0.766
UBC	1.065	1.601

B) Effect of combining HKG



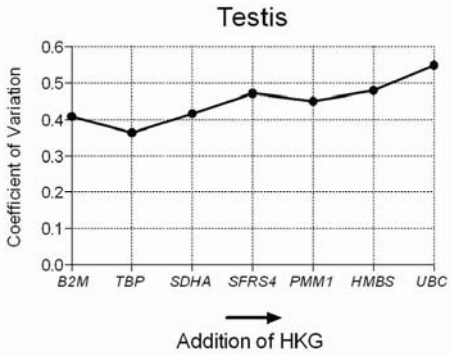
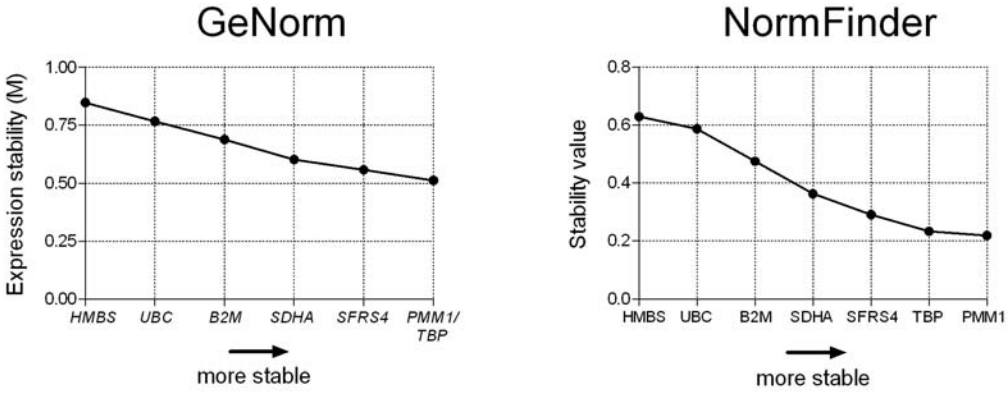


Figure 4

A) Ovary



B) Testis