

# Beta-Actin Cannot be Used as a Control for Gene Expression in Ovine Interstitial Cells Derived from Heart Valves

Jessa Yperman, Geoffrey De Visscher, Paul Holvoet, Willem Flameng

Cardiovascular Research Laboratory, Centrum voor Experimentele Heelkunde en Anaesthesiologie, Katholieke Universiteit Leuven, Leuven, Belgium

**Background and aim of the study:** In gene expression studies, endogenous controls that are constitutively expressed (housekeeping genes) are commonly used to normalize for variations in cDNA synthesis efficiency. In the present study, a frequently used control gene,  $\beta$ -actin, was examined in ovine heart valves to evaluate its applicability as a housekeeping gene for this tissue.

**Methods:** Interstitial cells (IC) of the four heart valves were isolated using the outgrowth explant method. Cells were cultured under different serum conditions (10% or 20% fetal bovine serum or 20% sheep serum) up to passage (P) 5. mRNA from fresh tissue and from cells at P<sub>0</sub> and P<sub>5</sub> was isolated, and expression of  $\beta$ -actin determined using reverse transcription-polymerase chain reaction (RT-PCR). An identical control sample was used for each PCR and each gel elec-

trophoresis. Data were expressed as a relative value of this control sample.

**Results:**  $\beta$ -Actin expression in the aortic valve was significantly lower than in other valves. The mRNA level of  $\beta$ -actin was four-fold lower in freshly isolated IC than in cultured IC. Once up-regulated by in-vitro culturing conditions,  $\beta$ -actin expression did not change from P<sub>0</sub> to P<sub>5</sub>. An important increase in the variation of  $\beta$ -actin expression was observed in cultured cells as compared to fresh cells. Different serum conditions did not lead to different  $\beta$ -actin levels.

**Conclusion:** Due to the variation in expression,  $\beta$ -actin cannot be used as a reference for gene expression of ovine-derived heart valve IC in culture.

The Journal of Heart Valve Disease 2004;13:848-853

Reverse transcription-polymerase chain reaction (RT-PCR) is an appropriate and common method to compare expression levels of mRNA of specific genes (1). For mRNA quantification, an internal control gene that is constitutively expressed - a so-called housekeeping gene - is used in most cases. This control gene is used to normalize the mRNA expression level which is necessary to correct for sampling differences in order to identify real variation in gene expression (2).

One interest in the present authors' laboratory is the study of cardiovascular and valvular disease, and the

main model used for valvular deterioration is that of a juvenile sheep; consequently, ovine interstitial heart valve cells form the current subject of investigation. The present authors are currently unaware of any validated housekeeping genes applicable in ovine interstitial heart valve cells. This validation is imperative, as normalization with a variably expressed gene would lead to misleading results (1-3). Consequently, the study aim was to evaluate the applicability of  $\beta$ -actin as an internal control for gene expression studies in ovine heart valve tissue and cells.

$\beta$ -Actin is one of the most frequently investigated control genes. By conducting a review of recent (1999) issues of high-impact journals, Suzuki et al. found the relative frequency for the use of  $\beta$ -actin as a control for mRNA quantification to be 32% (4). Nevertheless, other groups have recently demonstrated both instability and large variations in terms of gene expression of these so-called housekeeping genes in human tissues. Indeed, the results of these investigations in general state that ideal and universal control genes do not exist (2), and that  $\beta$ -actin is not constitutively expressed in human tissue (1-5).

---

Presented as a poster at the Second Biennial Meeting of the Society for Heart Valve Disease, 28th June-1st July 2003, Palais des Congrès, Paris, France

Address for correspondence:  
Prof. W. Flameng, Centrum voor Experimentele Heelkunde en Anaesthesiologie, Katholieke Universiteit Leuven, Provisorium 1, Minderbroedersstraat 17, 3000 Leuven, Belgium  
e-mail: Willem.Flameng@med.kuleuven.ac.be

## Materials and methods

### Animal surgery

The investigations conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996). Tissue samples were obtained from "Lovenaar" sheep sacrificed for non-interfering experiments approved by the ethical committee of the Katholieke Universiteit Leuven. These sheep were pure-bred in the animal farm of the K.U. Leuven, especially for experimental purposes, were never in contact with other animals, and were subjected to constant veterinary observation.

Sheep were premedicated with ketamine (10-20 mg/kg, intramuscularly) and anesthetized as described previously (6). Anesthesia was induced with increasing concentrations of halothane in oxygen (100%) by means of a mask, after which endotracheal intubation was achieved and mechanical ventilation started. Anesthesia was maintained with halothane (2%) in the breathing gas mixture (75% O<sub>2</sub>, 25% N<sub>2</sub>O). Fentanyl (Janssen Pharmaceutica, Beerse, Belgium) was administered as a bolus when necessary.

A left thoracotomy was performed in the second intercostal space, and the heart was dissected free from the surrounding tissues. To prevent blood clotting, heparin (3 mg/kg; Aventis Pharma, Brussels, Belgium) was administered and, after exsanguination, the heart was excised.

All four heart valves: aortic (A), mitral (M), tricuspid (T) and pulmonary (P) were harvested aseptically. For the isolation of RNA from tissue, a small piece (~15 mm<sup>2</sup>) of each valve was snap-frozen and stored in liquid nitrogen. The valve samples for cell isolation were stored in ice-cold saline solution (0.9% NaCl; Baxter, Lessines, Belgium) during transport prior to RNA isolation (less than 1h).

### Cell isolation and expansion

Interstitial cells were isolated from 108 pieces of ovine heart valve leaflets (n = 25 A, 27 M, 28 P and 28 T). Interstitial cell isolation was performed using the explant outgrowth technique (7). In brief, to prevent bacterial infection the tissue was first rinsed with Hanks' balanced salt solution (HBSS; Cambrex Biosciences, Verviers, Belgium) supplemented with antibiotic-antimycotic solution, containing 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Invitrogen, Merelbeke, Belgium). The endothelial layer was scraped away, the remaining valve tissue was minced into pieces of ~1 mm<sup>2</sup>, and the fragments were seeded into a collagen-coated 25-cm<sup>2</sup> flasks (Greiner, Wommel, Belgium) and cultured in Dulbecco's Modified Eagle Medium

(DMEM), containing 4.5 g/l glucose, antibiotic-antimycotic solution and serum in a humidified atmosphere with 5% CO<sub>2</sub>. Interstitial cells were cultured under different serum conditions: 10% fetal bovine serum (FBS, Invitrogen) (n = 6 A, 6 M, 7 P, 6 T), 20% FBS (n = 6 A, 8 M, 6 P, 8 T), and 20% sheep serum (SS) (n = 6 A, 6 M, 6 P, 6 T). During expansion, the medium was changed twice weekly and the first confluence was considered as passage 0 (P<sub>0</sub>). Confluent cells were detached by trypsinization (trypsin-EDTA; Invitrogen) and counted after trypan blue exclusion. Cells were subsequently expanded to passage 5 (P<sub>5</sub>).

### RNA isolation and two-step RT-PCR

Total RNA from the snap-frozen fresh tissue (n = 7 A, 7 M, 9 P, 8 T) was isolated by means of Trizol reagent (Invitrogen) according to the manufacturer's instructions. Trizol (800 µl) was added to the frozen tissue to maintain the integrity of the RNA while disrupting cells and dissolving cell components. Using a pestle and mortar, the tissue was crushed and mechanically homogenized (Polytron PT 10-35). Chloroform addition followed by centrifugation separated the solution into an aqueous phase (containing the RNA) and an organic phase. After transfer of the aqueous phase, the RNA was recovered by precipitation with isopropanol.

From the cell fractions in culture, total RNA was isolated from cells obtained at P<sub>0</sub> and P<sub>5</sub> confluence using the SNAP-kit (Invitrogen) according to the supplier's protocol. Briefly, 10<sup>6</sup> cells were homogenized in lysis-buffer to release RNA and inactivate RNases. The RNA and DNA of the cell lysate were extracted by means of a column filled with RNA- and DNA-retaining resin. The bound DNA-RNA was released from the resin using DNase/RNase-free water and treated with DNase to remove contaminating DNA. The RNA was put on a second column to eliminate DNase and degraded DNA.

For cDNA synthesis, the Thermoscriptkit (Invitrogen) was used starting from 1 µg total RNA with oligo(dT)<sub>20</sub> as primer. The cDNA was then used for PCR amplification of β-actin. Due to insufficient knowledge of ovine sequences, primers were designed based on homologous domains in human and mouse sequence using vector NTI software (Informax, North Bethesda, MD, USA). The primers were: F-5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA3' and R-5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG3' (661 bp).

PCR was performed in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM MgCl<sub>2</sub> in a volume of 50 µl. For amplification of the cDNA, 12.5 pmol of each primer, 0.125 mM of each deoxynucleoside triphosphate and 2.5 units of Taq polymerase (Takara, Cambrex Biosciences) were added to 2.5 µg

cDNA. The PCR was conducted in a thermocycler (PTC-200; Biozym, Langraaf, The Netherlands). Denaturation (1 min, 95°C) was followed by 30 cycles each consisting of a denaturation cycle at 94°C for 10 s, annealing at 55°C for 10 s and extension at 72°C for 30 s. The final step was an extension of 10 min at 72°C. For each sample, two PCR reactions were performed independently. Aliquots of the amplified DNA were placed on an ethidium bromide (0.5 µg/ml) -stained agarose (1%) gel (21 × 19.5 cm) and the electrophoresis was run at 135 V for 90 min using a Tris-borate-EDTA buffer containing 0.2 µg/ml ethidium bromide. The gel was visualized by ultraviolet transillumination and then analyzed by densitometry using Quantity One software of the Gel Doc 2000 (Bio-Rad, Nazareth, Belgium).

The cDNA of one sample was divided into small aliquots. An aliquot of this sample served as an internal control for each PCR and each gel electrophoresis. All data were expressed as a relative value compared to this control sample. To confirm the results, some samples (1 µl) of the amplified DNA were placed on a DNA 1000 chip for the 2100 bioanalyzer (Agilent Technologies, Diegem, Belgium) according to the manufacturer's instructions. This technology uses gel-filled channels that allow for molecular sieving by applying electrokinetic forces. This results in an electropherogram where the area under the peak is a measure of β-actin expression. The electropherogram obtained can be visualized as a gel-like image.

#### Data analysis

All results were expressed as medians and 95% confidence intervals (CI). An unpaired multiple group comparison was performed by means of a Kruskal-Wallis analysis. A two-sided Wilcoxon-Mann-Whitney rank-sum test was used for analysis between groups separately. A two-sided p-value <0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, Illinois, USA).

## Results

#### Optimization and validation of PCR measurements

Buffer and primer concentrations were optimized by evaluating the PCR-product on an agarose gel. Conditions for amplification were established that gave a single amplified band of the predicted size (661 bp). Afterwards, a dilution series of two samples was prepared to determine the range of the initial cDNA concentration where PCR amplification was linear. In the range from 0.25 to 2 µg cDNA/10 µl, the Pearson correlation coefficient R<sup>2</sup> was 0.860 for one sample, and 0.998 for the other sample. Therefore, an initial con-

centration of 0.5 µg cDNA/10 µl was chosen.

The gray intensity range in which measurements by densitometry were correct was also validated, and hence a dilution series of a sample was made after amplification. The initial concentration was 0.5 µg cDNA/10 µl and, after amplification, the product was

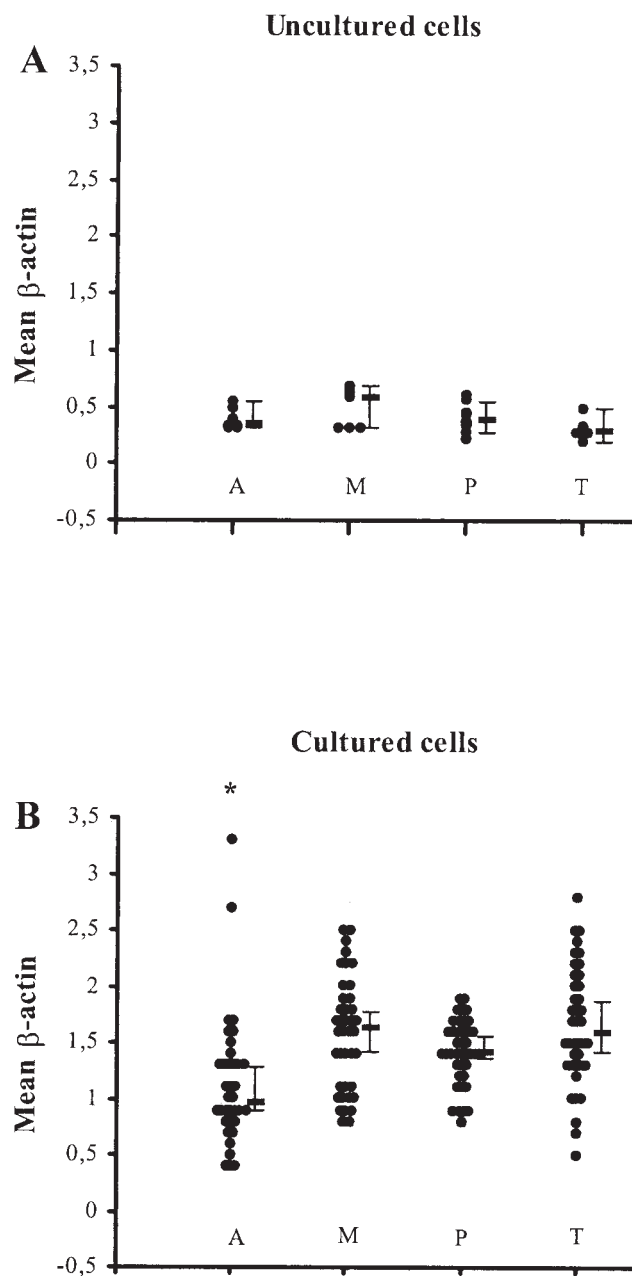


Figure 1: β-Actin expression in interstitial heart valve cells, grouped by valve type. The uncultured cells (panel A) may contain up to 10% endothelial cells, but are mainly interstitial cells. The cultured cells (panel B) are purely interstitial cells. Optical density values are expressed as relative value compared to control sample. A: Aortic; M: Mitral; P: Pulmonary; T: Tricuspid. \*Significantly different (p < 0.05) compared to other groups.

measured undiluted and then diluted from 1:2 to 1:128. Plotting the results showed that, starting from 0.5 µg cDNA/10 µl, the amplification of β-actin was in a range where detection was linear ( $R^2 = 0.9975$ ).

### Reproducibility of measurements

Following cDNA synthesis in the first step of the RT-PCR, cDNA was divided in aliquots and two aliquots of one sample were used for PCR amplification and

agarose gel electrophoresis. Therefore, two independent measurements were available for each of the 187 samples. The mean error between both measurements was 8.5%.

### Homogeneity of the studied cells

Due to the sampling method used for freshly isolated cells (mRNA-isolation from snap-frozen tissue), it was not possible to discard the endothelial cells from the interstitial cells. Counting endothelial cells and interstitial cells on hematoxylin and eosin-stained native ovine aortic valves showed endothelial cells to comprise 9.8% of the total cell number. Hence, the freshly isolated cell fractions, containing approximately 90% interstitial cells and 10% endothelial cells, were hereafter referred to as freshly isolated interstitial cells.

### β-Actin expression in heart valve interstitial cells

β-Actin expression in the different valve interstitial cells is presented in Figure 1. The expression was stable over the four different valves in freshly isolated cells (Fig. 1A). β-Actin expression in cultured aortic valve interstitial cells was significantly lower than in other valves (Fig. 1B). The expression was 1.68-fold higher in mitral, 1.64-fold higher in tricuspid, and 1.46-

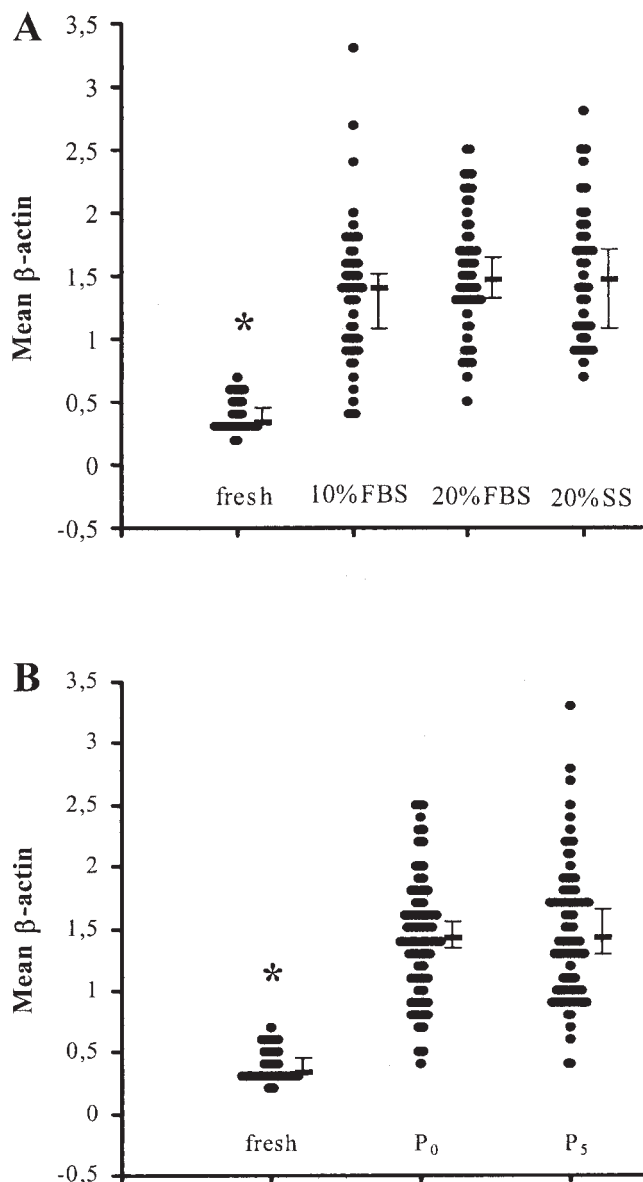


Figure 2: β-Actin expression in interstitial heart valve cells, grouped by serum condition during expansion (panel A) and by expansion passage (panel B). Optical density values are expressed as relative value compared to control sample. P<sub>0</sub>: First passage; P<sub>5</sub>: Passage 5; fresh: Freshly isolated interstitial cells. \*Significantly different ( $p < 0.05$ ) compared to other groups.

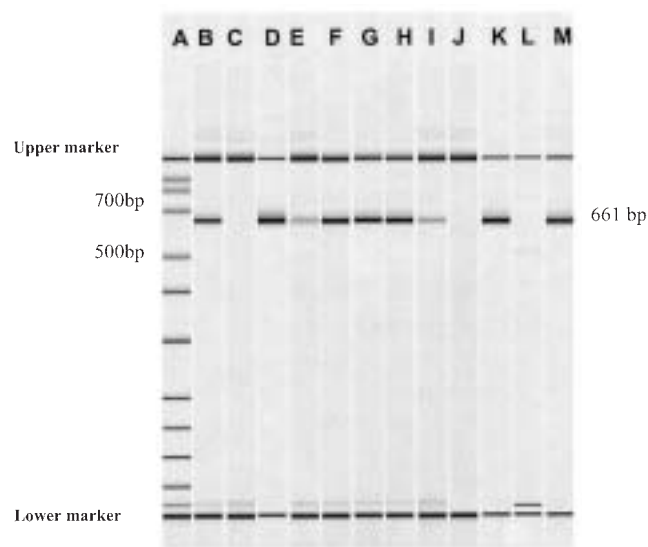


Figure 3: Gel-like image of an electropherogram obtained with the 2100 bioanalyzer. The expression of β-actin (PCR fragment of 661 bp) is not equal in all samples; however the initial concentration of cDNA was the same in all samples. Lane identification: A: Ladder; B: Positive control (sheep ventricle); C: Negative control; D: Control sample; E-M: samples, E: Tricuspid fresh; F: Aortic fresh; G: Mitral 1 fresh; H: Mitral 2 fresh; I: pulmonary fresh; J: Aortic 1 P<sub>0</sub> 10% FBS; K: Aortic 2 P<sub>0</sub> 10% FBS; L: Aortic P<sub>0</sub> 20% SS; M: Mitral P<sub>0</sub> 10% FBS. bp: Base pairs.

fold higher in pulmonary valves. The  $\beta$ -actin mRNA level was significantly lower in freshly isolated interstitial cells than in cultured interstitial cells (Fig. 2A and 2B). Different serum conditions did not result in different  $\beta$ -actin levels, but  $\beta$ -actin expression was increased 4.07-fold in 10% FBS, 4.24-fold in 20% FBS, and 4.24-fold in 20% SS compared to freshly isolated cells.  $\beta$ -Actin expression increased 4.14-fold in P<sub>0</sub> and 4.11-fold in P<sub>5</sub> compared to freshly isolated cells, but did not change from P<sub>0</sub> to P<sub>5</sub>. Furthermore, an important increase in the variation of  $\beta$ -actin expression was observed in cultured interstitial cells as compared to fresh interstitial cells. An analysis of data from Figure 2A and 2B within each valve type showed the same result. Fresh valve cells showed a significantly lower  $\beta$ -actin expression compared to cells in culture in all four valve types. Additionally, for tricuspid valve interstitial cells  $\beta$ -actin expression was significantly higher in 20% SS compared to 10% FBS.

When the PCR product was applied to an electrophoresis-chip to measure  $\beta$ -actin expression, the results of the agarose gel electrophoresis and densitometry were confirmed. An example of a gel-like image acquired by the bioanalyzer is shown in Figure 3, and indicates that  $\beta$ -actin expression was not equal in all samples.

## Discussion

In heart valve research, the juvenile sheep model is the most important large animal model for preclinical testing (6,8), and at the present authors' laboratory, valve characteristics are investigated using this model on a cellular level. Therefore, gene expression studies of ovine heart valve-derived interstitial cells both in fresh isolates and under culture conditions are required. Unfortunately, the genetic background of this animal model is rather poor compared to, for example, human and mouse. As the authors were unaware of any validated housekeeping gene in the juvenile sheep model, a study was conducted on the applicability of  $\beta$ -actin as an internal control for gene expression. Although frequently used in several areas of research, the validity of  $\beta$ -actin - a structural protein of the cytoskeleton - as a housekeeping gene has recently been questioned. Warrington et al. (5) studied gene expression levels in different human adult and fetal tissues based on high-density oligonucleotide arrays, and concluded that  $\beta$ -actin expression is turned on early in fetal development and stays on throughout adulthood. However, the expression level was found to vary up to 22-fold between different tissues. An evaluation by Vandesompele et al. (2) of 10 housekeeping genes in various human tissues measured by real-time RT-PCR showed that  $\beta$ -actin was among the

worst-scoring genes with regard to stability of expression. In a search for a stable control gene in differentiating T helper cells, Hamalainen et al. (3) found an 11.4-fold change in  $\beta$ -actin expression with an increased expression during T-cell differentiation, measured using real time RT-PCR. Levels of transcription were also seen to vary in different porcine tissues (9) and in canine myocardium (10).

The most striking finding of the present study was the four-fold increase in  $\beta$ -actin expression in cultured interstitial cells compared to freshly isolated interstitial cells. Furthermore, this was associated with an important increase in the variation of  $\beta$ -actin expression in cultured interstitial cells compared to fresh cells. The difference in  $\beta$ -actin expression between freshly isolated and cultured interstitial cells cannot be the consequence of the 10% of endothelial cells in the freshly isolated cells. If the endothelial cells were unable to express  $\beta$ -actin at all, a 1.11-fold difference in  $\beta$ -actin expression would be expected, but in reality the difference was more than four-fold. A study conducted by Lowe et al. (11) showed that when serum-free cultured rat fibroblasts were incubated for 18 h with 1% FBS,  $\beta$ -actin mRNA levels increased four-fold. Therefore serum incubation might cause the differential  $\beta$ -actin expression in cultured ovine heart valve interstitial cells. A possible cause of different  $\beta$ -actin expression in freshly isolated and cultured interstitial cells might be the quiescent life within normal valve tissue, which contrasts greatly with the increased mitotic state during expansion. The changes in  $\beta$ -actin expression cannot be accounted for by differences in RNA isolation, as the optical density measurements at wavelengths of 260 nm and 280 nm were comparable between the freshly isolated and cultured interstitial cells. The initial cDNA concentration (0.5  $\mu$ g/10  $\mu$ l) was also based on spectrophotometric measurements at 260 nm. The differences in expression cannot be attributed to any differences in PCR amplification or gel electrophoresis efficiency as all samples were run in random sequence.

In the absence of a validated housekeeping gene for ovine heart valve interstitial cells, gene expression studies on these cells can be carried out using one identical sample as a control for each PCR and each gel electrophoresis. Data should be expressed as a relative value compared to this control sample. This method is straightforward and easy to apply compared to searching a valuable housekeeping gene. Although it would be very interesting for this field of research to have at its disposal a gene that is expressed equally in uncultured and cultured cells and also during different phases of the cell cycle, the methodology for normalization described herein offers an appropriate alternative, beside more complex PCR methods (e.g. internal control by mimic RNA) (12).

In conclusion, the results of the present study have demonstrated that  $\beta$ -actin expression in ovine heart valve interstitial cells is increased in cultured cells compared to freshly isolated cells. However, due to this variation in expression,  $\beta$ -actin cannot be used as a housekeeping gene for ovine-derived heart valve interstitial cells in culture.

#### Acknowledgements

The authors thank Veerle Leunens for assistance in sample collection, and Ruth Plusquin for technical assistance in cell culturing and cell staining. These investigations were supported by the Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen (G.0231.01) and the Katholieke Universiteit Leuven (OT TBA).

#### References

1. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-193
2. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.
3. Hamalainen HK, Tubman JC, Vikman S, JA et al. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001;299:63-70
4. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. *Biotechniques* 2000;29:332-337
5. Warrington JA, Nair A, Mahadevappa M, Tsyganskaya M. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol Genomics* 2000;2:143-147
6. Flameng W, Ozaki S, Yperman J, et al. Calcification characteristics of porcine stented valves in a juvenile sheep model. *Ann Thorac Surg* 2001;71:S401-S405
7. Rizvi MAD, Katwa LC, Spadone DP, Myers PR. The effects of endothelin-1 on collagen type I and type III synthesis in cultured porcine coronary artery vascular smooth muscle cells. *J Mol Cell Cardiol* 1996;28:243-252
8. Barnhart GR, Jones M, Ishihara T, Rose DM, Chavez AM, Ferrans VJ. Degeneration and calcification of bioprosthetic cardiac valves. Bioprosthetic tricuspid valve implantation in sheep. *Am J Pathol* 1982;106:136-139
9. Foss DL, Baarsch MJ, Murtaugh MP. Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. *Anim Biotechnol* 1998;9:67-78
10. Carlyle WC, Toher CA, Vandervelde JR, McDonald KM, Homans DC, Cohn JN. Changes in beta-actin mRNA expression in remodeling canine myocardium. *J Mol Cell Cardiol* 1996;28:53-63
11. Lowe WL, Jr., Kummer M, Karpen CW, Wu XD. Regulation of insulin-like growth factor I messenger ribonucleic acid levels by serum in cultured rat fibroblasts. *Endocrinology* 1990;127:2854-2861
12. Haberhausen G, Pinsl J, Kuhn CC, Markert-Hahn C. Comparative study of different standardization concepts in quantitative competitive reverse transcription-PCR assays. *J Clin Microbiol* 1998;36:628-633