A comprehensive survey of the laminins and collagens type IV expressed in mouse Leydig cells and their regulation by LH/hCG

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Abstract

Extracellular matrix (ECM) proteins have been shown to alter Leydig cell steroidogenesis in vitro, substantiating the hypothesis that Leydig cell steroidogenic activity and matrix environment are interdependent events. However, the nature of the ECM components synthesized by Leydig cells and their regulation by LH/human chorionic gonadotropin (hCG) remain unknown. Here, we examine the occurrence of the 11 laminin subunits and the 6 α chains of collagen IV (COL4A1–6) by RT-PCR in Leydig cells cultured with or without LH/hCG. Leydig cells were a tumor Leydig cell line (mLTC-1) or 8-week-old mice Leydig cells. Based on PCR data, it is suggested that normal Leydig cells may synthesize a maximum of 11 laminin heterotrimers and the 6 α chains of collagen IV. They also may synthesize various proteases and inhibitors of the metzincin family. The mLTC-1 cells have a limited repertoire as compared with normal Leydig cells. Interestingly, none of the ten proteases and inhibitors monitored is under LH–hCG regulation whereas every protease and inhibitor of the serine protease family yet identified in Leydig cells is under gonadotropin regulation. In addition, a few laminin and collagen subunit genes are regulated by LH/hCG in both Leydig cell types, and Col4a4, which was downregulated in primary cultures but not in mLTC-1 cells.

Collectively, the present study suggests that Leydig cells modulate in a selective fashion their matrix environment in response to their trophic hormone. This may alter the steroidogenic outcome of Leydig cells.


Introduction

Collagen type IV, laminins, nidogens, and heparan sulfate proteoglycans are essential components of basement membranes (BMs). BMs are specialized structures of the extracellular matrix (ECM) with multiple functions, including proliferation, migration, survival, and differentiation (Timpl 1996, Timpl & Brown 1996). Collagen IV is essential for BM stability. It is composed of three α chains assembled in a triple helix. They are six different chains of collagen type IV, α1–α6, each encoded by a separate gene, but only three specific protomers, α1α1α2, α3α4α5, and α5α5α6 (Timpl 1996). While α1(IV) and α2(IV) chains are ubiquitously present in BMs, the α3–α6 (chains) generally have a more restricted distribution (Hudson et al. 2003, Zheng et al. 2005).

Laminins are large heterotrimeric glycoproteins consisting of α, β, and γ chain, encoded by specific genes named Lama, Lamb, and Lamc respectively. To date, five α chains, three β chains, and three γ chains have been identified, from which 16 laminin heterotrimers have been characterized in vivo (Aumailley et al. 2005). Laminin can bind to collagen IV, entactin/nidogen, heparin sulfate proteoglycan and itself, and is thought to have an organizing role in matrix assembly and the resulting supramolecular architecture (Colognato & Yurchenko 2000). Thus, the composition of BMs can be highly variable depending on the types of laminin and collagen type IV, and it is now recognized that the unique composition of each BM contributes to its specific functional properties. In addition, its composition may change during development and growth, or in response to injury or an environmental or hormonal stimulus (Enders et al. 1995, Kahsai et al. 1997, Pöschl et al. 2004). Changes in BM composition result from modifications in the balance between ECM component synthesis and degradation, and two major groups of enzymes are...
involved in ECM component degradation, that is, the enzymes of the plasminogen activator (PA) system and the family of matrix metalloproteinases (MMPs; Dano et al. 2005, Page-McCaw et al. 2007).

The testis is divided into two compartments with the seminiferous tubules that produce sperm cells and the interstitial compartment that contains the steroidogenic Leydig cells. In the testis, the biological functions and composition of the BM surrounding the seminiferous tubules have been the subject of many investigations, and its importance is highlighted with the finding that male infertility is associated with abnormal thickening in the BM underlying the seminiferous epithelium (de Kretser et al. 1975). Indeed, the BM is the structural basis of testis cord development (Dym 1994, Clark et al. 2000). In adult life, it is essential for the maintenance of the differentiated functions of Sertoli cells that are to provide structural and nutritional support for the developing germ cells (Grissold 1998, Siu & Cheng 2004).

Conversely, little is known on the composition and functional importance of the ECM components of Leydig cell origin. It is known that Leydig cells are not surrounded by a continuous BM in the adult testis, but that they exhibit patches of laminin and type IV collagen on the cell surface. A direct association of these ECM proteins with Leydig cells has also been evidenced (Kuopio & Pelliniemi 1989). Indeed, ECM affects Leydig cell proliferation, testosterone production, and gene expression (Vernon et al. 1991, Diaz et al. 2002, 2005). However, the nature of the ECM components synthesized by Leydig cells and their regulation by the trophic hormone luteinizing hormone (LH) have remained unexplored.

In the present study, we show at the RNA level that Leydig cells synthesize various laminins and collagens type IV subunits and a few are downregulated by LH/human chorionic gonadotropin (hCG) via cAMP. Leydig cells also synthesize various proteinases and inhibitors, but none of the molecules here studied are under LH/hCG regulation. Taken collectively with our previous study reporting a rapid and transient induction of PA of the urokinase-type in cultured Leydig cells upon LH stimulation (Odet et al. 2006), these data indicate that Leydig cells can selectively modulate their matrix environment in response to LH/hCG.

**Results**

**RT-PCR screening of laminins and collagens type IV**

The list and sequences of the designed specific primers for PCR studies are described in Table 1. Lamb3 is not presented because it is not expressed in the mouse testis (Häger et al. 2005). Total RNA was recovered from adult mouse testes, 3-week-old Sertoli cells that had been cultured for 2 days in basal conditions, 8-week-old freshly isolated Leydig and mLTC-1 cells. A PCR product of the right size and sequence (not shown) was detected for each of the molecules studied using total RNA from adult testes or Sertoli cells. PCR using specific primers directed against Sox9 was used to detect Sertoli cell contamination of the 8-week-old Leydig cells.

The expression pattern of the laminin α subunits was different between normal Leydig and mLTC-1 cells. Normal Leydig cells exhibited a PCR product for every laminin α subunit with the exception of the Lama1 subunit, whereas mLTC-1 cells only expressed the Lama1, Lama3 (weakly), and Lama4 (Fig. 1). The two Leydig populations exhibited a PCR product for Lamb1, Lamb2, Lamc1, and Lamc3. Lamc2 was absent in mLTC-1 cells and weakly present in normal Leydig cells (Fig. 1). Since there was a weak Sox9 PCR band in normal Leydig cells and no band for Lamc2 in the mLTC-1 cells (Fig. 1), it is likely that the weak band of Lamc2 detected in the 8-week-old Leydig cells resulted from the weak contamination of the Leydig cell preparations with Sertoli cells expressing Sox9. Other PCR signals for laminin subunits had roughly similar intensities in Sertoli and Leydig cell preparations (Fig. 1). Thus, normal Leydig and mLTC-1 cells may be able to synthesize a maximum of 11 and 7 laminin heterotrimerics respectively (Table 2).

Regarding the six α chains of collagen IV, the two populations exhibited a PCR product for Col4a2–6. The signal for Col4a2–5 was weaker in mLTC-1 cells than in normal Leydig cells. The signal for Col4a6 was weak in both Leydig cell types. Normal Leydig cells also exhibited a PCR product for Col4a1 (Fig. 1). Based on PCR data, it sounds unlikely that PCR signals in normal Leydig cells result from the weak contamination of the Leydig fraction with Sertoli cells. These data indicate that normal Leydig cells are probably able to synthesize the three specific protomers α1α2α2, α3α4α2, and α5α5α6, whereas mLTC-1 cells can only synthesize the trimers α3α4α5 or α5α5α6 (Table 2).

**Addition of hCG alters the expression of ECM proteins in Leydig cells**

In these experiments, the expression of StAR was monitored to ensure that Leydig cells responded significantly (P<0.05) to hCG and βu-cAMP (shown in the mLTC-1 cells) in our experimental conditions (Fig. 2). For the semi-quantitative RT-PCR experiments, we ran 29 cycles for Lama3 and Lamc3 (Fig. 2), and 25 cycles for the Col4a3, Col4a4, and Col4a6 (Fig. 3). The signals detected by RT-PCR using total RNA from mLTC-1 cells were weaker than the signals generated with total RNA from 8-week-old Leydig cells, indicating a relative low abundance of the transcripts in the tumoral cells (Figs 2 and 3).

Most of the laminins (Lama1, Lama2, Lama4, Lama5, Lamb1, Lamb2, Lamc1, and Lamc2) and collagen (Col4a1, Col4a2, and Col4a5) subunit genes were not regulated by LH/hCG (not shown). Conversely, laminins α3 and γ3 (Fig. 2), and Col4a3, Col4a4, and Col4a6 (Fig. 3) were...
significantly \((P<0.05)\) downregulated by LH/hCG in primary cultures, with a fold decrease ranging from \(-30\) to \(-60\%\) depending on the molecule, and when compared with time-matched controls. Effects were time dependent. In the case of \(\text{Lama}3\), \(\text{Col4a}3\), and \(\text{Col4a}4\), first significant effects \((P<0.05)\) were detected after 2 h and maintained at 6 and 24 h of stimulation. \(\text{Lamc}3\) and \(\text{Col4a}6\) were also significantly \((P<0.05)\) downregulated by hCG after 24 h of stimulation but no significant effect was detected at earlier times (Figs 2 and 3).

The effects observed in mLTC-1 cells were very similar to those described in normal Leydig cells with the exception of \(\text{Col4a}4\), which was not regulated in mLTC-1 cells (Fig. 3). However, the kinetics of the effects was slightly different depending on the molecule. For example, in mLTC-1 cells, \(\text{Lama}3\) was not regulated after 2 h of hCG stimulation. \(\text{Lamc}3\) was significantly downregulated \((P<0.05)\) as soon as 6 h of stimulation (Fig. 2). \(\text{Col4a}4\) was transiently downregulated after 6 h of hCG stimulation and \(\text{Col4a}6\) was downregulated after 6 and 24 h of hCG stimulation (Fig. 3).

The addition of 1 mM \(\text{bu}_2\text{cAMP}\) was found to mostly mimic the effects induced by hCG (shown for the mLTC-1 cells), indicating the involvement of the protein kinase A, even though little variation was observed. For example, \(\text{Lama}3\) was significantly downregulated by \(\text{bu}_2\text{cAMP}\) from 2 h onwards (Fig. 2). \(\text{Col4a}3\) was not significantly downregulated with \(\text{bu}_2\text{cAMP}\), although a tendency existed at 6 h consistent with the hCG downregulation of \(\text{Col4a}3\) at 6 h of stimulation (Fig. 3). A similar situation was described for \(\text{Col4a}6\), and no significant reduction of \(\text{Col4a}6\) was observed at 24 h of stimulation with \(\text{bu}_2\text{cAMP}\) (Fig. 3).

In order to further validate the semi-quantitative data, real-time PCR was carried out using \(\text{Rpi}19\) as a housekeeping gene. Data presented in Figs 2D and 3D indicate
that StAR was upregulated (a twofold increase; \( P < 0.05; n = 3 \)) following a hCG treatment of 24 h in 8-week-old Leydig cells. Using the same series of samples, we observed a significant (\( P < 0.05; n = 3 \)) downregulation of \( \text{Lama3} \), \( \text{Col4a3} \) (a 4-fold decrease), \( \text{Col4a4} \) (a 2.5-fold decrease), and \( \text{Col4a6} \) (a 7-fold decrease) in the 8-week-old Leydig cells treated for 24 h with hCG (100 ng/ml). Lower sensitivity of the semi-quantitative PCR versus real-time PCR probably explains differences in the fold changes.

**RT-PCR screening of various proteases and inhibitors and hCG regulation**

The list and sequences of the designed specific primers for PCR studies are described in Table 1. Samples studied are as described in Fig. 1. A PCR product of the right size and sequence (not shown) was detected for each of the molecules studied using total RNA from adult testis or Sertoli cells (Fig. 4). Leydig and mLTC-1 cells displayed a PCR product for \( \text{Mmp3} \) and \( \text{Mmp14} \). No PCR product was found for the gelatinases \( \text{Mmp2} \) and \( \text{Mmp9} \) in mLTC-1 cells. There was a weak band for \( \text{Mmp2} \) but no band for \( \text{Mmp9} \) in 8-week-old Leydig cells. The weak \( \text{Mmp2} \) band may result from the low contamination of the Leydig cell preparations with Sertoli cells (Fig. 1). Indeed, no gelatinolytic activity has ever been detected using Leydig cell-concentrated culture media (Odet et al. 2006).

A PCR product corresponding to tissue inhibitor of MMPs (\( \text{Timps} \))1–4 was also evidenced in the 8-week-old Leydig cells whereas mLTC-1 cells had a PCR product for \( \text{Timp1} \), \( \text{Timp3} \), and \( \text{Timp4} \) but not for \( \text{Timp2} \) (Fig. 4). The two Leydig populations exhibited a PCR product for cathepsin L (\( \text{Ctsl} \); Mathur et al. 1997), and a disintegrin and metalloproteinase domain (\( \text{Adam} \))21 (originally named \( \text{Adam31} \) in Liu & Smith 2000), as expected. None of the proteases or inhibitors present in Leydig cells were under LH–hCG regulation at the time-points investigated (2, 6, and 24 h; not shown).

**Discussion**

In the present study, we have investigated the *in vitro* occurrence of the laminins and collagen type IV subunit genes in adult Leydig cells and a tumoral Leydig cell line, and their regulation by gonadotropins. We have also examined whether various proteases and inhibitors specifically of the matrix metalloproteinase family are expressed in these cells and under a regulation by gonadotropins. The rationale for this study stems from previous observations. First, Leydig cells produce less testosterone upon LH stimulation when cultivated in plates coated with ECM components (Diaz et al. 2002, 2005). Secondly, Leydig cells have contacts with various ECM components (Fawcett et al. 1973), although they do not synthesize a continuous BM (Kuopio & Pelliniemi 1989). Thirdly, Leydig cells respond to LH *in vitro* by a transient burst of urokinase, a matrix degrading enzyme, concomitantly with an enhanced StAR expression (Odet et al. 2006). Collectively, these data highly suggest that a link exists between the capacity of Leydig cells to produce testosterone and their matrix environment.
In a first series of experiments using an RT-PCR procedure, we show the presence of mRNAs encoding several laminins and collagens type IV, MMPs, and TIMPs, and we evidence a different repertoire between the tumoral and the normal Leydig cells. Differences observed relate to the identity of the molecules, their relative abundance and gonadotropin regulation. Specifically, tumoral cells express a smaller repertoire than normal cells. In addition, some laminins or collagens IV are present (at least at the RNA level) exclusively in one but not in the other cell type. We also show (although this was not quantified) a weaker level of expression of the ECM components in the mLTC-1 cells as compared with normal Leydig cells. We also evidence that normal Leydig cells are weakly contaminated with Sertoli cells. Thus, it is possible that differences account for the tumoral phenotype of the mLTC-1 cells.

Normal Leydig cells have a PCR product for Lama2 whereas the mLTC-1 cells exhibit a PCR product for Lama1. Laminin α1 and α2 chains may play comparable functions since laminin α1 chain corrects male infertility caused by the absence of laminin α2 chain (Häger et al. 2005). Interestingly, LAMA3 and LAMC3 are downregulated by hCG, although with a kinetics slightly different depending on the laminin subunit and the cell type. Thus, provided laminin subunits are translated and assembled into proteins, three and five laminins potentially synthesized would be under LH–hCG control in mLTC-1 and normal Leydig cells respectively. LAMC3 is unique because it is located in murine BMs (Gersdorff et al. 2005), and mouse Leydig cells express LAMC3 (Iivanainen et al. 1999; this study). Conversely, it is associated with non-BM structures in the rat (Koch et al. 1999), such as the apical ectoplasmic specializations in adult rat testes (Yan & Cheng 2006).

Regarding collagens IV, mLTC-1 cells exhibit a PCR product for the α2–6 chains of collagen IV but no signal is evidenced for the α1 chain contrasting with normal Leydig cells. In addition, the regulation of the α3, α4, and α6 chains of collagen IV by hCG is different between the two cell types, in terms of the kinetics of downregulation of the Col4a3 and Col4a6 by hCG, and also Col4a4 is not regulated by hCG in the mLTC-1 cells. These differences may well be related to the different properties of these cells and the tumoral nature of the mLTC-1 cells. Indeed, the α1α1α2 protomer is essential for structural integrity of BMs and of BM-like structures (Pöschl et al. 2004). Interestingly, the protomers α3α4α5 and α5α5α6, but not the protomer α1α1α2, are potentially under gonadotropin regulation in the normal Leydig cells.

Few studies have explored the hormonal regulation of ECM components. To date, differential expression of several laminins and α chains of collagen has been shown in mice models with testicular androgen deficiency (Petruz et al. 2005, Denelet et al. 2006, Wang et al. 2006, Zhang et al. 2006). Other experiments conducted in vitro have shown that thyroid hormones on Sertoli cells (Ulisse et al. 1998) and estrogens on Leydig cells (Yashwanth et al. 2006) can regulate some ECM components. However, an in silico analysis reveals no consensus sequence for CRE in the 5’ untranslated promoter region of the regulated laminins and collagen subunit genes (not shown). Furthermore, it is possible that the pattern of ECM expression and regulation by hormones varies with the differentiation status of Leydig cells during development and in adult life.

In addition to studying the hormonal regulation of the laminin and the collagen subunit genes, we have examined if the Mmps and Timps identified in Leydig cells as well as Adam21 and cathepsin L previously reported to be present in Leydig cells (Mathur et al. 1997, Liu & Smith 2000) were under LH–hCG regulation. ADAM21 is a cell-surface protein which exhibits metalloproteinase activity (Primakoff & Myles 2000) and cathepsin L belongs to the cysteine family of protease and is under FSH regulation in Sertoli cells (Penttila et al. 1995). We observed no regulation of these molecules by hCG. Given that every serine proteases and serine protease inhibitors yet identified in Leydig cells are

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under gonadotropin regulation, and that urokinase is elevated rapidly and transiently following LH/hCG stimulation (Odet et al. 2006), our data may suggest that gonadotropins regulate the expression of proteases and inhibitors in a selective manner. Specifically, it is possible that proteases, such as urokinase, exert a primary role in Leydig cells. It is a matrix degrading enzyme with a large spectrum of activities towards most of ECM molecules (Dano et al. 2005). In addition, proteases are generally synthesized as proenzymes and urokinase can process...
them to an active form directly or through a cascade of sequential activation (Dano et al. 2005, Page-McCaw et al. 2007). In that scheme, urokinase upon LH/hCG stimulation will initiate a cascade of proteolytic events, and the various proteases expressed in Leydig cells will be activated, thus altering local matrix environment, not only through the degradation of ECM molecules synthesized by their own but also through ECM molecules originating from endothelial cells. Indeed, Leydig cells are stabilized within the interstitium through attachments

Figure 3 Effect of hCG and bu2cAMP on the expression levels of (A) Col4a3, (B) Col4a4, and (C) Col4a6 on primary cultures of Leydig cells isolated from 8-week-old testes and in mLTC-1 cells, assessed using a semi-quantitative RT-PCR procedure. Leydig and mLTC-1 cells were cultured for 2, 6, or 24 h with or without 100 ng/ml hCG or 1 mM bu2cAMP. Autoradiographs were scanned and expression level was normalized to the Hprt1 signal. The values correspond to the mean ± S.E.M. of n = 3–5 independent experiments of triplicates (Leydig cells) or n = 4–5 independent experiments of duplicates (mLTC-1). Asterisks indicate significant differences relative to untreated cells of the time-matched control (Leydig cells) or control mLTC-1 cells (C) (P < 0.05, Kruskal–Wallis ANOVA followed by Dunn's test). A representative gel of one experiment and the corresponding Hprt1 signal are presented.

(D) Effect of a 24-h hCG treatment on the expression levels of Col4a3, Col4a4, and Col4a6 on primary cultures of Leydig cells isolated from 8-week-old testes, assessed using real-time PCR. The Rpl19 levels-normalized values correspond to the mean ± S.E.M. of n = 3 independent experiments of duplicates, and asterisks indicate significant differences relative to untreated cells of the time-matched control Leydig (P < 0.05, t-test).
made to BM components that surround blood vessels and lymphatic endothelium (Fawcett et al. 1973, Kuopio & Pelliniemi 1989, Vernon et al. 1991). Collectively, our data raise the hypothesis that the positive regulation exerted by LH–hCG on steroidogenesis is the result of two phenomena. A direct and well-described effect of LH–hCG on steroidogenesis through binding to its receptor and activation of target genes and enhancement of certain steroidogenic enzymes including StAR (Stocco et al. 2005), and an indirect pathway that is to prevent the inhibiting role exerted by the matrix environment on the steroidogenic capacity of Leydig cells. For example, it has been shown that type IV collagen induces downregulation of gonadotropin response to gonadotropins in adult rat Leydig cells (Diaz et al. 2005). Such inhibition will be exerted at two levels. First, there will be a selective inhibition of ECM synthesis, as shown in the present study. Secondly, there will be an enhancement of the matrix degrading enzyme urokinase (Odet et al. 2006, Le Magueresse-Battistoni 2007), and urokinase will degrade ECM molecules directly or through the activation of the other proteases present locally. Future studies will have to be designed to determine the soundness of such a hypothesis.

Materials and Methods

Animals, tissues, and cell preparations

Swiss male CD-1 mice, aged 3 and 8 weeks, were purchased from Elevage Janvier (Le Genest, France). Animals were killed by CO₂ asphyxia before the removal of testes. Testes were immediately used for cell preparations or stored at −70 °C for RNA analysis. Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals.

Sertoli cells were isolated from 3-week-old mice testes and cultured in HAM’s F12–DMEM (Life Technologies) at 32 °C in a humidified atmosphere of 5% CO₂ as described previously (Le Magueresse-Battistoni et al. 1998). At the end of the enzymatic procedure, the Sertoli cell suspension was washed with fresh culture medium and cells were seeded in six-well plates at a ratio of 1 × 10⁶ viable cells/well in Ham’s F12–DMEM. They were cultured for 2 days. At that time, the purity of the cultures was higher than 90%, and contamination was mainly due to residual germ cells (an average of 5–7%; not shown).

Leydig cells were isolated from 8-week-old mice testes and cultured in HAM’s F12–DMEM at 32 °C in a humidified atmosphere of 5% CO₂ as described previously (Carreau et al. 1988). Briefly, testes were decapsulated and digested with 0.25 mg/ml collagenase at 32 °C for 10 min. The digestion procedure was stopped by dilution with fresh medium, followed by two successive washes and centrifugations. Interstitial cells were purified on a discontinuous Percoll density gradient (layers of 21, 26, 43, 40, and 60% Percoll). The gradient was centrifuged at 800 g for 30 min. The interface between 40 and 60% was collected and washed with medium to remove the Percoll. The presence of 3-β hydroxysteroid dehydrogenase activity was revealed by a histochemical technique described in details elsewhere (Bilinska et al. 1997). It was used to determine the purity of Leydig cells, which ranged from 90 to 95%. Cells were resuspended in fresh culture medium supplemented with 2% fetal calf serum. They were plated in 12-well plates (150 000 cells/well). An aliquot of freshly isolated Leydig cells was also processed for RNA analysis.

Cell line cultures

The immortalized Leydig cell mLTC-1 line was kindly provided by DM Stocco (Texas Tech, Lubbock, TX, USA). The mLTC-1 cells were cultivated at 37 °C in a humidified 5% CO₂ incubator. Culture medium was a RPMI 1640 medium (Sigma–Aldrich Corp.) supplemented with 10% fetal calf serum until cells reach sub-confluency (70%), as described (Manna et al. 2004). Serum was then omitted, cells were rinsed abundantly, and fresh medium was replaced.

Figure 4  RT-PCR analysis of various proteases and inhibitors in mLTC-1 cells, freshly isolated Leydig cells recovered from 8-week-old testes, Sertoli cells recovered from 3-week-old testes which had been cultured for 5 days in basal conditions, and 8-week-old mice testes. The set of primers used is listed in Table 1. RT-PCR studies were also conducted using primers directed against Hprt1 to ensure that equal amounts of material were used. A DNA ladder was included in each gel for accurate determination of the size of the PCR product and each amplicon was sequenced (not shown).
Cell treatment
In the relevant experiments, 100 ng/ml hCG; (Organon, Puteaux, France) or 1 mM bu2cAMP (Sigma) diluted in culture medium were added to the cultures. For the mLTC-1 cells, stimulation was performed 24, 6, and 2 h before cultures were terminated, whereas primary Leydig cells were stimulated 24 h after plating for 2, 6, and 24 h.

RNA extraction, RT-PCR, and semi-quantitative RT-PCR
Procedure for RNA extraction and RT-PCR has been described elsewhere (Longin et al. 2001, Guyot et al. 2003). Briefly, specific primers were designed using the Gene Jockey sequence processor (Biosoft, Cambridge, Cambbs, UK) and primer3 software, and the optimal temperature of annealing was defined for each couple of primers (Table 1). Negative controls contained water instead of cDNA. PCR with no RT reactions gave no product, eliminating the possibility of a genomic DNA contamination in the RNA preparations. Amplified cDNAs were visualized in a 1.5% agarose gel stained with ethidium bromide. A DNA ladder (Promega) was loaded on each gel and Hprt1 (hypoxanthine–guanine phosphoribosyl transferase) was used to ensure equal loading. PCR products were sequenced by Biofidal (Lyon, France). PCR product sequences were found to match the published reference ratio of the calibrator.

Quantitative PCR
Real-time PCR was carried out using the LightCycler DNA Master SYBR Green I kit (Roche Diagnostics GmbH) in a LightCycler Instrument (Roche Diagnostics GmbH). The PCR was performed with 0.4 μM of each primer, between 3 and 5 nM MgCl₂, 1.5 μl LightCycler DNA Master SYBR Green I in a total volume of 20 μl. After the initial denaturation step of 8 min at 95 °C, the reaction conditions were 45 cycles of 95 °C for 15 s, 60 or 64 °C (depending on the primer; Table 1) for 5 s, and 72 °C for 8–16 s. The fluorescence intensity of SYBR Green was read on the LightCycler after the end of each extension step. Melting curve analyses were performed immediately following the final PCR cycle to verify the specificity of the PCR product by looking at its Tm. Rpl19 gene was chosen as the reference for normalizing the samples. It was consistently and reproducibly expressed in all samples, and it did not vary following hCG treatment (data not shown). Relative quantification was made by the standard curve method for both target and housekeeping gene (endogenous control) in each sample. A series of dilutions of calibrator sample (external standard) was included in each experiment in order to generate an external standard curve. Then the concentration of the target in each sample was divided by the concentration of the housekeeping gene in each sample, thereby normalizing the samples. Relative quantification was carried out using the LightCycler Relative Quantification Software (version 1.0). The calculation of data was based on the crossing point values obtained by the LightCycler Software (Roche Diagnostics GmbH). To correct for sample heterogeneity and variability of detection, the results were calculated as the target/reference ratio of the sample divided by the target/reference ratio of the calibrator.

Statistical analysis
All experiments made on duplicates or triplicates have been performed at least thrice with independent preparations of cells. All values are the mean ± S.E.M. of 3–7 points, as precised in the text or in the figures. The significance of the results of semi-quantitative PCR data was examined by Kruskal–Wallis one-way ANOVA on ranks (comparison between several groups) followed by Dunn’s test (multiple comparisons versus control group). The significance of the results of quantitative PCR data was examined by t-test. P<0.05 was considered significant. All statistical analyses were done with the aid of the Sigmatstat 3.1 software package (Systat Software Inc., Point Richmond, CA, USA).

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