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

Séverine Mazaud Guittot, Adélie Vérot, Fanny Odet¹, Marie-Agnès Chauvin² and Brigitte le Magueresse-Battistoni

Inserm U418, UCBL1, UMR INRA 1245, Hôpital Debrousse, 29 rue soeur Bouvier, 69322 Lyon Cedex 05, France, ¹NIH NIEHS 111 TW Alexander Drive Research Triangle Park, Research Triangle Park, North Carolina 27709, USA and ²INSERM U870 INRA U1235, Faculté de Médecine Lyon Sud, 165 Chemin du Grand Revoyet, 69600 Oullins, France

Correspondence should be addressed to S Mazaud Guittot; Email: severinemazaud@yahoo.fr; lemagueresse@lyon.inserm.fr

A Vérot and F Odet contributed equally to this work
S Mazaud Guittot and B le Magueresse-Battistoni are now at INSERM U870 INRA U1235, Faculté de Médecine Lyon Sud, 165 Chemin du Grand Revoyet, 69600 Oullins, France

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. These are laminins $\alpha 3$ and $\gamma 3$ (*Lama3* and *Lamc3*), *Col4a3*, and *Col4a6*, which are negatively 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.

Reproduction (2008) **135** 479–488

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, $\alpha 1$ – $\alpha 6$, each encoded by a separate gene, but only three specific protomers, $\alpha 1\alpha 1\alpha 2$, $\alpha 3\alpha 4\alpha 5$, and $\alpha 5\alpha 5\alpha 6$ (Timpl 1996). While $\alpha 1$ (IV) and $\alpha 2$ (IV) chains are ubiquitously present in BMs, the $\alpha 3$ – $\alpha 6$ (chains) generally have a more restricted distribution (Hudson *et al.* 2003, Zheng *et al.* 2005). Laminins are large heterotrimeric glycoproteins consisting of a 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 organization at the time of testis differentiation in the developing gonad (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 (Griswold 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). In addition, 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 mice 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 heterotrimers 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 $\alpha1\alpha1\alpha2$, $\alpha3\alpha4\alpha5$, and $\alpha5\alpha5\alpha6$, whereas mLTC-1 cells can only synthesize the trimers $\alpha3\alpha4\alpha5$ or $\alpha5\alpha5\alpha6$ (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 bu_2cAMP (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 $\alpha3$ and $\gamma3$ (Fig. 2), and *Col4a3*, *Col4a4*, and *Col4a6* (Fig. 3) were

Table 1 List and sequence of the designed specific primers for PCR studies.

	Accession no.	Primers (5'-3')		Size (bp)	T _{Opt} (C)
		Forward	Reverse		
<i>Hprt1</i>	NM_013556	CCTGCTGGATTACATTAAGCACTG	GTCAGGGCATATCCAACAACAAAC	354	65
<i>Rpl19</i> ^a	NM_009078	CTG AAG GTC AAA GGG AAT GTG	GGA CAG AGT CTT GAT GAT CTC	195	60
<i>StAR</i>	NM_011485	GCATACTCAACAACCAGGAAGG	CTGGTTGATGATTGTCTTCGGC	511	65
<i>StAR</i> ^a		TTGGGCATACTCAACAACCA	CCTTGACATTTGGGTTCCAC	198	60
<i>Sox9</i>	AF421878	TCC AGC AAG AAC AAG CCA CAC G	CTT GTC CGT TCT TCA CCG	270	58
Laminins					
<i>Lama1</i>	NM_008480	TTACGTGGGTGGATTGCCTCAT	TGTTCTGTCCATCACTGTAGG	449	61
<i>Lama2</i>	NM_008481	GTATGGTCTTTGAAAATACAAC	GATTCTGCTCTGGAGTTGGTCC	206	60
<i>Lama3</i>	XM_140451	TTGAAGGCACAGGCTATGCTCG	TGTATCGTTCAACCTGACAACC	454	65
<i>Lama3</i> ^a		AAATGCTCCCTTCCCAAAC	ATCTCTCCCGTTGTGATGG	193	60
<i>Lama4</i>	NM_010681	TGCTCAAACCAGGAGTGTC	CACCTTTCCTGTTTCCCTA	378	65
<i>Lama5</i>	NM_001081171	CACCGAAGTGGTCTATTCTGGC	CCTACGCTTACATTGACACTCC	446	65
<i>Lamb1</i>	NM_008482	GTACATTGGTCCAGATGTGAT-GACT	GGCAATGGTCCCTTCTGTGTGGT	184	65
<i>Lamb2</i>	NM_008483	AGAAGGCAGAGACAGTCCAAGC	GTATTGGTCACCTACTTGTTC	328	60
<i>Lamc1</i>	NM_010683	GTGTGAACGATAACAAGACAGC	CATACCGTTACCTCATTATCC	268	62
<i>Lamc2</i>	NM_008485	TCGTATCAGCAGCTCTCCG	GCAACCTTCTGGCTAATAGAGG	421	62
<i>Lamc3</i>	NM_011836	TTGCAGGGCAGACACTCGTT	GTCTCCAGTCTTACTACTACG	226	61
Collagens					
<i>Col4a1</i>	NM_009931	TATCTCTGGGGACAACATCCG	CATCTCGTTCTCTCTATGGTG	321	65
<i>Col4a2</i>	NM_009932	GCCACTACTTCGCTAACAAAG	GACAGACGGTTGGAATGAGTGC	469	65
<i>Col4a3</i>	NM_007734	ATTAGTGGCAGAGCTCTCGAAC	CATTCTTTCTGGGTTGAGCGAAG	320	65
<i>Col4a4</i>	NM_007735	TGCGGCTCCTCTTCTATGAT	GCTGGGCCTGAACCTCTTTC	397	65
<i>Col4a4</i> ^a		CTCTGGTTCTCCACAGTCAG	AAGGGCAGAGTGCTAAACACA	167	64
<i>Col4a5</i>	NM_007736	TAGCCGATGTCAAGTATGC	TTGCTCTGGCACATTGCTGAGG	250	61
<i>Col4a6</i>	NM_053185	TCGTGTCTTGAGGACTTCCG	TGTTAGCACCAGATGGATGG	419	61
<i>Col4a6</i> ^a		AGAAAGCCCATACACAGGATCT	GCTGATATACTGGGGAACTGG	194	60
Proteases					
<i>Adam21</i>	NM_020330	CACCTACACAGAGGAACACACC	ACTTCTTAGCCTCTTCAACTCC	310	61
Cathepsin L Cts/	NM_009984	ATGAGGAAGAGTGGAGGAGAGC	GTCATTAGCCACAGCGAACTCG	541	68
<i>Mmp2</i>	NM_008610	CTATTCTGTGACGACTTTGG	CAGACTTTGGTTCTCAACTT	309	58
<i>Mmp3</i>	NM_010809	ACTTACCCTCAGCCCAAGG	TCCAGAGAGTTAGACTTGGTGG	348	61
<i>Mmp9</i>	NM_013599	TGTTAGCAAGGGGCGTGTC	AAACAGTCCAACAAGAAAGG	461	63
<i>Mmp14</i>	NM_008608	CTGATGACGATCGCCGTGGCATCC	GCGTCTGAAGAAGAAGACAGCGAGG	880	65
Inhibitors					
<i>Timp1</i>	NM_011593	CTGGCATCTCTTGTGCTA	AGGGATCTCCAGGTGCACAA	585	56
<i>Timp2</i>	NM_011594	ATCAGAGCCAAAGCAGGTGAGCG	GGTAATGTGCATCTTGCCATCTCC	243	69
<i>Timp3</i>	NM_011595	CCA GAC AAG AGC ATC AGC	ACTTCTGCCAACTTCCTATCC	481	63
<i>Timp4</i>	NM_080639	TTT GAC CAT CAC CAC CTG CTG T	ACT TCA GCC AAA CAG CCC ACT C	375	69

The optimal temperature (T_{Opt}) for annealing, the size of the expected PCR fragments, and the source for the design of the primers are reported. The number of cycles was determined for each set of primers. It ranged from 21 to 30.

^aPrimers designed specifically for real time quantitative PCR.

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 *Lama3*, *Col4a3*, and *Col4a4*, first significant effects ($P < 0.05$) were detected after 2 h and maintained at 6 and 24 h of stimulation. *Lamc3* and *Col4a6* 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 *Col4a4*, 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, *Lama3* was not regulated after 2 h of hCG stimulation. *Lamc3* was significantly downregulated ($P < 0.05$) as soon as 6 h of stimulation

(Fig. 2). *Col4a3* was transiently downregulated after 6 h of hCG stimulation and *Col4a6* was downregulated after 6 and 24 h of hCG stimulation (Fig. 3).

The addition of 1 mM bu_2cAMP 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, *Lama3* was significantly downregulated by bu_2cAMP from 2 h onwards (Fig. 2). *Col4a3* was not significantly downregulated with bu_2cAMP , although a tendency existed at 6 h consistent with the hCG downregulation of *Col4a3* at 6 h of stimulation (Fig. 3). A similar situation was described for *Col4a6*, and no significant reduction of *Col4a6* was observed at 24 h of stimulation with bu_2cAMP (Fig. 3).

In order to further validate the semi-quantitative data, real-time PCR was carried out using *Rpl19* as a house-keeping gene. Data presented in Figs 2D and 3D indicate

Figure 1 RT-PCR analysis of various laminins and collagen subunit genes 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, and against *Sox9* to assess the purity of Leydig cells. 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).

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 *Lama3*,

Lamc3, *Col4a3* (a 4-fold decrease), *Col4a4* (a 2.5-fold decrease), and *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 *Mmp3* and *Mmp14*. No PCR product was found for the gelatinases *Mmp2* and *Mmp9* in mLTC-1 cells. There was a weak band for *Mmp2* but no band for *Mmp9* in 8-week-old Leydig cells. The weak *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 (*Timps*)1–4 was also evidenced in the 8-week-old Leydig cells whereas mLTC-1 cells had a PCR product for *Timp1*, *Timp3*, and *Timp4* but not for *Timp2* (Fig. 4). The two Leydig populations exhibited a PCR product for cathepsin L (*Ctsl*; Mathur *et al.* 1997), and a disintegrin and metalloproteinase domain (*Adam*)21 (originally named *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 collagens 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.

Table 2 List of extracellular matrix (ECM) molecules potentially synthesized by Leydig cells based on PCR studies.

Laminin subunits	$\alpha 1$		$\alpha 2$		$\alpha 3$		$\alpha 4$		$\alpha 5$		$\beta 1$		$\beta 2$		$\beta 3$		$\gamma 1$		$\gamma 2$		$\gamma 3$			
mLTC-1	+	—	—	—	+	+	+	+	—	—	+	+	+	+	—	—	+	+	—	—	+	+		
8-week-old Leydig cells	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	—	+	+		
Laminin isoforms	111	211	121	221	332	311	321	411	421	511	521	213	423	522	523									
mLTC-1	+	—	+	—	—	+	+	+	+	—	—	—	+	—	—									
8-week-old Leydig cells	—	+	—	+	—	+	+	+	+	+	+	+	+	—	+									
Collagen IV subunits	$\alpha 1$				$\alpha 2$				$\alpha 3$				$\alpha 4$				$\alpha 5$				$\alpha 6$			
mLTC-1	—				+				+				+				+				+			
8-week-old Leydig cells	+				+				+				+				+				+			
Collagen IV protomers	$\alpha 1\alpha 1\alpha 2$								$\alpha 3\alpha 4\alpha 5$								$\alpha 5\alpha 5\alpha 6$							
mLTC-1	—								+								+							
8-week-old Leydig cells	+								+								+							

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 $\alpha 1$ and $\alpha 2$ chains may play comparable functions since laminin $\alpha 1$ chain corrects male infertility caused by the absence of laminin $\alpha 2$ 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 (Gersdoff *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 $\alpha 2$ – $\alpha 6$ chains of collagen IV but no signal is evidenced for the $\alpha 1$ chain contrasting with normal Leydig cells. In addition, the regulation of the $\alpha 3$, $\alpha 4$, and $\alpha 6$

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 $\alpha 1\alpha 1\alpha 2$ protomer is essential for structural integrity of BMs and of BM-like structures (Pöschl *et al.* 2004). Interestingly, the protomers $\alpha 3\alpha 4\alpha 5$ and $\alpha 5\alpha 5\alpha 6$, but not the protomer $\alpha 1\alpha 1\alpha 2$, 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 collagens has been shown in mice models with testicular androgen deficiency (Petrusz *et al.* 2005, Denolet *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 (Penttilä *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

Figure 2 Effect of hCG and bu₂cAMP on the expression levels of (A) *StAR*, (B) *Lama3*, and (C) *Lamc3* 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 $\pm$ S.E.M. of $n=6$ independent experiments of triplicates (Leydig cells) or $n=3-7$ independent experiments of duplicates (mLTC-1 cells). 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 *StAR*, *Lama3*, and *Lamc3* 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 $\pm$ 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).

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

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 $\pm$ 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 $\pm$ 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).

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 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).

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 steroidogenic 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 1×10^6 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.

Cell treatment

In the relevant experiments, 100 ng/ml hCG; (Organon, Puteaux, France) or 1 mM bu_2cAMP (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, Cambs, 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 sequences (not shown). Conditions for reliable semi-quantitative RT-PCR were optimized for each series of primers in the presence of α -33P dATP (0.75 μCi ; 2500 Ci/mmol; Amersham Pharmacia Biotech Europe GmbH), as described elsewhere (Guyot *et al.* 2003, Odet *et al.* 2004). The PCR products were separated on 8% PAGE in 1 $\times$ Tris–borate–EDTA buffer. Gels were transferred to filter paper, dried, and exposed to Kodak biomax MR1 films (Sigma). The band densities were determined by scanning densitometric analysis (Scion image beta 4.03; Scion Corporation at www.scioncorp.com). Densitometry data were normalized using *Hprt1*.

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 MgCl_2 , 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 *T_m*. *Rpl19* gene was chosen as the reference for normalizing target gene. 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 $\pm$ 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 Sigmasat 3.1 software package (Systat Software Inc., Point Richmond, CA, USA).

Acknowledgements

We are indebted to Douglas M Stocco (Texas University, Lubbock, TX, USA) for providing us with the mLTC-1 cells. We are grateful to Catherine Rey (Profilexpert, Lyon) for carrying out real-time PCR experiments. This work was supported by Inserm U418 (Institut National de la Santé et de la Recherche Médicale), INRA UMR 1245 (Institut National de la Recherche Agronomique), and by the University of Lyon Claude Bernard (UCBL1). Adélie Vérot was funded by Organon (Azko, Nobel). Fanny Odet was funded by MRT (Ministère de la Recherche et de la Technologie) and FRM (Fondation pour la Recherche Médicale).

S Mazaud Guittot, A Vérot, F Odet, M A Chauvin and B le Magueresse-Battistoni have no conflict of interest to declare.

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Received 15 December 2007
First decision 21 January 2008
Accepted 21 January 2008