

Fine Temporal Analysis of DHT Transcriptional Modulation of the ATM/Gadd45g Signaling Pathways in the Mouse Uterus

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SUMMARY

In rodents, the uterus of a mature female undergoes changes during the uterine cycle, under the control of steroid hormones. 5α -Dihydrotestosterone (DHT) is recognized to play an important role in the regulation of androgen action in normal endometrium. Using microarray technology, a screening analysis of genes responding to DHT in the uterus of ovariectomized mice, has allowed us to highlight multiple genes of the *ATM/Gadd45g* pathway that are modulated following exposure to DHT. Two phases of regulation were identified. In the early phase, the expression of genes involved in the G2/M arrest is rapidly increased, followed by the repression of genes of the G1/S checkpoint, and by the induction of transcriptional regulators. Later, *i.e.* from 12 to 24 hr, genes involved in G2/M transition, cytoarchitectural and lipid-related genes are stimulated by DHT while immunity-related genes appear to be differentially regulated by the hormone. These results show that a physiological dose of DHT induces the transcription of genes promoting the cell cycle progression in mice. Profile determination of temporal uterine gene expression at the transcriptional level enables us to suggest that the DHT modulation of genes involved in *ATM/Gadd45g* signaling in an ATM- or p53-independent manner, could play an important role in the cyclical changes of uterine cells in the mouse uterus.

Mol. Reprod. Dev. 76: 278–288, 2009. © 2008 Wiley-Liss, Inc.

Received 17 December 2007; Accepted 26 May 2008
By Ralph Gwatkin as Editor in Chief

Published online 31 July 2008 in Wiley InterScience
(www.interscience.wiley.com).
DOI 10.1002/mrd.20949

INTRODUCTION

The cyclical changes of the endometrium are under the control of steroid hormones including estrogens, progesterone and androgens. While the estrogenic effect is well documented in mouse uterus, the androgen action on uterine physiology is poorly defined. Actions of active androgens (testosterone and dihydrotestosterone) are mediated by the androgen receptor (AR), a transcription factor member of the nuclear receptor family. The enzyme 5α -reductase catalyzes the conversion of testosterone (T) to the more potent agonist 5α -dihydrotestosterone (DHT). Considering that during the secretory phase in which both AR and 5α -

reductase are present in endometrial epithelial cells (Ito et al., 2002; Pelletier et al., 2004) and given its higher binding affinity for AR (Martel et al., 1998), DHT might play a more important role than T in the androgen action on normal endometrium. Androgens are known to exert both proliferative and anti-proliferative effects in human and mice. Administration of T or DHT has been shown to increase uterine weight of immature hypophysectomized rats (Armstrong and Papkoff, 1976) and DHT alone has also been reported to induce proliferation of endometrial

Additional supporting information may be found in the online version.



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epithelial and myometrial smooth muscle cells, as well as mammary ductal and stromal cells in mice (Zhang et al., 2004).

On the other hand, opposite observations have shown that androgens demonstrate an antiestrogenic activity in mammary epithelial cells of human and Rhesus monkeys (MacIndoe and Etre, 1981; Zhou et al., 2000) as well as in rat uterus (Hung and Gibbons, 1983). Interestingly, there is evidence of an existing dose-related antagonistic effect of DHT on estrogenic effects in the pig uterus (Càrdenas and Pope, 2004). High levels of endometrial androgen are also associated with miscarriage and infertility (Okon et al., 1998; Tuckerman et al., 2000). Indeed, genes involved in cell cycle regulation that would be responsible for these opposite effects of DHT observed in apoptosis or stimulation of uterine epithelial cells, remain to be identified.

In mammals, DNA repair, checkpoint activity and apoptotic processes are the major cellular responses induced by DNA damage (Niida and Nakanishi, 2006). ATM is one of the sensor proteins employed by the DNA damage checkpoints to detect DNA damage and to initiate signal transduction cascades involving several kinases and phosphatases. ATM, as signal transducer, can activate p53 and inactivate cyclin-dependent kinases (CdKs) to inhibit the G1/S, the intra-S or the G2/M checkpoints, which will allow DNA repair processes (Sancar et al., 2004). *Gadd45* is one of the transcriptional target of p53 implicated in the regulation of the G2/M transition (Taylor and Stark, 2001). Activation of *Gadd45* leads to the inhibition of the kinase activity of the Cdc2a/CyclinB1 complex required for the G2/M progression (Vairapandi et al., 2002). However, a p53-independent activation of *Gadd45* is also reported (Jin et al., 2001). Indeed many *Gadd45*-interacting proteins have been identified, namely Oct-1, NF-YA (Jin et al., 2001), WT1 (Zhan et al., 1998), p21, pCNA, Cdc2 (Yang et al., 2000), Brca1 (Jin et al., 2000) and particularly MTK1 (Nakayama et al., 1999), which is an activator of the Jun N-terminal kinase and p38 MAP kinase pathways (Lu et al., 2001).

While G1 pathway defects (*PTEN*, *CyclinD1* and *p27*) have been associated with the progression of endometrial hyperplasia (EH) to endometrial cancer (EC) in human, only few reports exist regarding the role of the ATM-CHK2-CDC25C-CDC2/CyclinB1 pathway (known as the G2 pathway) in uterine physiology. Loss of CHK2 protein expression has been observed in few EC samples (Tsuda et al., 2003), and *CHK1* has been proposed to have a limited role in EC (Semba et al., 2000). Although *ATM* protein expression does not show any significant modulation in EC, altered protein expression of other components of the G2 pathway were more often observed in EC than EH samples (Tsuda et al., 2003). *GADD45A* gene expression has been reported to be higher in the secretory phase than the proliferative phase of the endometrium (Borthwick et al., 2003), and given that *GADD45* proteins (α , β and γ) share similar functions, this is in agreement with the inhibitory role of these proteins in cell cycle progression. Moreover, higher *GADD45* expression has also been observed in human during the implantation window, which corresponds to the mid-secretory phase that is clearly distinct from the proliferative phase (Hertig and Rock, 1956).

The aim of the present study is to analyze the impact of DHT on uterus gene expression of ovariectomized mice using microarray technology in establishing an exhaustive temporal gene expression profile over a 24-hr period to evaluate the potential role of DHT on the regulation of major components of the cell cycle in an estrogen-dependent tissue such as the uterus. As expected, DHT appears to induce, during this period, the transcription of genes whose products are involved in the promotion of the cell cycle and the early activation of *Gadd45g* gene (specific of G2/M arrest), suggesting that these regulations could play a pivotal role in the cyclical changes of uterine cells in the mouse.

RESULTS

Microarray Analysis: Genomic Response of the Uterus to DHT

To study the time course impact of DHT on uterus physiology, we analyzed 237 DHT-regulated uterine genes (Supplemental Table 1). These selected genes, whose regulation is stimulated or repressed upon six different profiles (A–F), are represented in Figure 1. For the

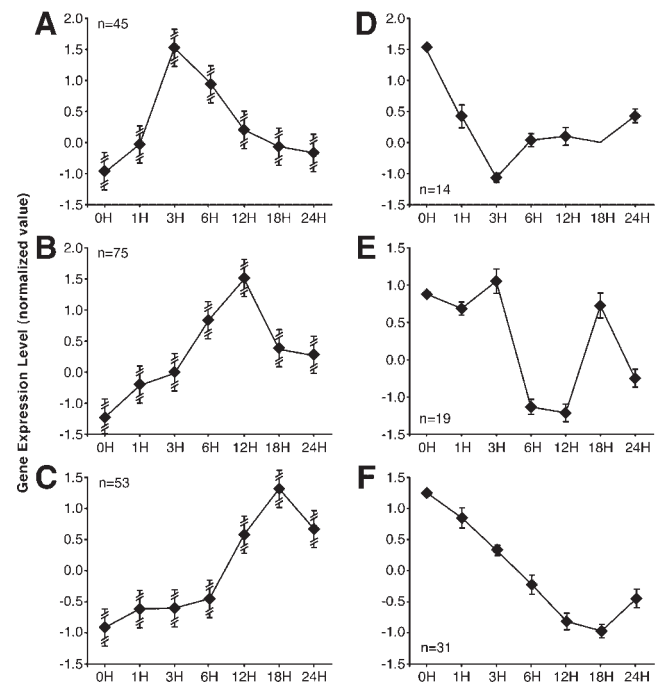


Figure 1. Clustering of gene expression patterns. Among 237 genes that passed the variation filter, the 173 up-regulated genes and the 64 down-regulated genes were normalized to obtain a mean = 0 and a S.D. = 1 across time points, separately. After the normalization, using a self-organizing map (GeneCluster 2), they were grouped into three clusters (A–C) and three clusters (D–F) of activated and inhibited genes, respectively. Each cluster is represented by the centroid of normalized values on the Y-axis and time points on the X-axis. The number of genes belonging to each cluster is displayed. These patterns indicate the representative temporal gene expression changes of the selected genes. Error bars indicate the maximal and minimal fold value for the panels A–C and the S.D. of the average expression levels for the panels D–F.

clustering analysis we used (1×3) node numbers for the DHT-induced and down-regulated genes. These node numbers are representative of the temporal change of the genes listed for the clustering, because they give independent clustering patterns. Most DHT-modulated genes are stimulated (73%, 173 out of 237). We found 3 expression profiles among these 173 increased genes: genes of cluster A (26%, 45 out of 173) were induced within 3 hr, genes of cluster B (43%, 75 out of 173) are up-regulated within 12 hr and genes of cluster C (31%, 53 out of 173) within 18 hr. The majority of genes stimulated by DHT are represented in cluster B. Similarly, 3 expression profiles appear within the 64 genes that are down-regulated by the hormone. Genes of cluster D (22%, 14 out of 64) were repressed within 3 hr, genes of cluster E (30%, 19 out of 64) showed a repression plateau between 6 hr and 12 hr, while genes of cluster F (48%, 31 out of 64) were down-regulated within 18 hr. The majority of DHT-decreased genes are found in cluster F.

Following their expression pattern profile, genes from each cluster (A–F) were then categorized into 10 different biological processes using the L2L and EASE softwares depicting an overview of major gene functions represented within each cluster during the time frame of 24 hr. The genes not assigned to a known function were classified as unknown biological process. Although no statistical value could be associated with this classification, the gene proportion of each function for a given profile has been calculated and is illustrated in Figure 2. According to the data presented on this figure, it can be seen that, among the stimulated genes (clusters A–C), genes involved in signal transduction and transcription, as well as in stress and immunity are mainly represented in cluster A. The genes involved in the energy and protein metabolism are more represented in cluster B, the latter group being clearly dominant in this cluster. The major gene functions found in cluster C are associated with transport and development, and genes involved in

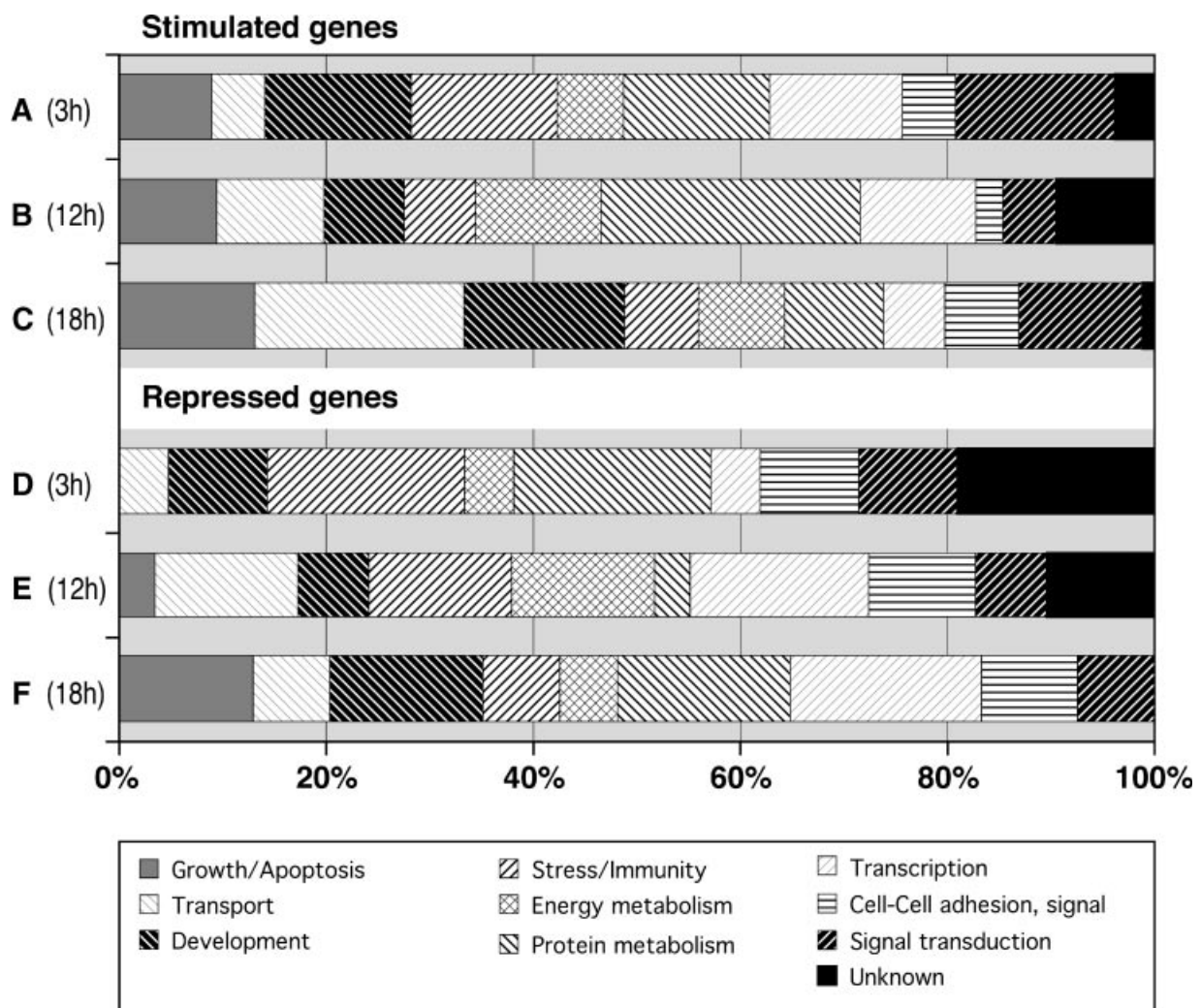


Figure 2. Functional classification of clustered genes. Different functions of the clustered genes were grouped into 10 categories using L2L and EASE softwares and the percentage representation of each function is indicated. Each corresponding cluster is indicated on the left as well as the peak of regulation for each cluster in parentheses.

growth and apoptosis are also over-represented in this cluster.

Among the down-regulated genes represented here (clusters D–F), we found that genes related to stress and immunity are dominant in cluster D, along with the genes involved in protein metabolism. The stress/immunity group is also dominant in cluster E, with genes related to the transcription, transport and energy. In cluster F, over-representation of some genes related to growth/apoptosis, development, protein metabolism and transcription is observed.

Specific Up-Regulated Genes: 0–24 hr (Clusters A–C). Some of the specific subsets of genes regulated by DHT are illustrated in Figure 3, while the whole dataset can be found in Supplemental Table 1. To avoid any duplication of information, only certain subfunctions are represented.

Genes associated to signal transduction and transcription are predominantly up-regulated in cluster A, as shown in Figure 2, especially genes belonging to mRNA catabolism and ribosome biogenesis, which demonstrate a FE of 30.7 (data not shown) and 13.3, respectively. Specific oncogenes not associated to any over-represented

subfunctions (*i.e.* not included in Fig. 3), such as *surfeit gene 2* or *homeo box A11*, also appear to be increased during this phase (clusters A and B), whereas some are repressed, such as *c-Jun* (cluster E) (Supplemental Table 1). Furthermore, genes involved in the response to stress and pregnancy display a FE of 3.1 and 14.0, respectively. Among them, *Gadd45g* is involved in the cellular defense program and in G2/M arrest. Similarly, other genes related to G2/M checkpoint such as *Wee1* and *Stratifin* (14-3-3 protein), are induced within 3 hr by DHT (cluster A) (Supplemental Table 1). Moreover, the *PLZF* gene, known for its anti-proliferative activity, is also strongly up-regulated (fivefold) at 3 hr (Supplemental Table 1).

Genes associated to protein folding or response to unfold protein, show a FE of 6.4 and 13.1 (data not shown), and protein transport-related genes are also over-represented in cluster B, presenting a FE of 14.6. At a later stage, genes implicated in cell division are principally induced (cluster C), displaying a FE of 13.4, and include specific genes involved in the G1 phase (FE = 20.5), control point of mitotic cell cycle (FE = 52.8) and mitosis (FE = 10.6) (data not shown). For instance, *Rad51*, a gene involved in DNA repair, is up-regulated, as well as *CyclinA2* and *Cdk4*, two genes associated with the G1/S transition, and certain genes of the G2/M transition such as *Cdc2a*, *CyclinB1-2/Ccnb1-b2* and *Cdc25c*, are all induced within 18 hr. Genes involved in cell organization and biogenesis (FE = 2.1, data not shown), and organ development related-genes (FE = 3.7) are also principally induced during this phase (cluster C), along with tissue development (FE = 5.2, data not shown) and tissue morphogenesis-related genes (FE = 16.8, data not shown). Many genes implicated in transport processes are also slightly over-represented in this phase (FE = 2.0).

Specific Down-Regulated Genes: 0–24 hr (Clusters D–F). Although no specific biological process could be clearly identified in cluster D, due mainly to the limited number of genes, the gene *SCPEP1*, whose product is involved in proteolysis (serine carboxypeptidase activity), is one member of this cluster (Supplemental Table 1).

In cluster E, we found inhibition of genes involved in the positive regulation of cell differentiation, which showed a FE of 41.5, and particularly myeloid cell differentiation-related genes presenting a FE of 24.4 (data not shown). Some genes involved in protein targeting (FE = 10.8), namely *Uhmk1* and *Rhoa*, are both members of this group of genes. Cluster E also includes genes involved in oxidative stress response, which display a FE of 23.0. The cluster F comprises genes implicated in a positive regulation of apoptosis (FE = 6.4), particularly *Iff16*, which is also involved in immune response.

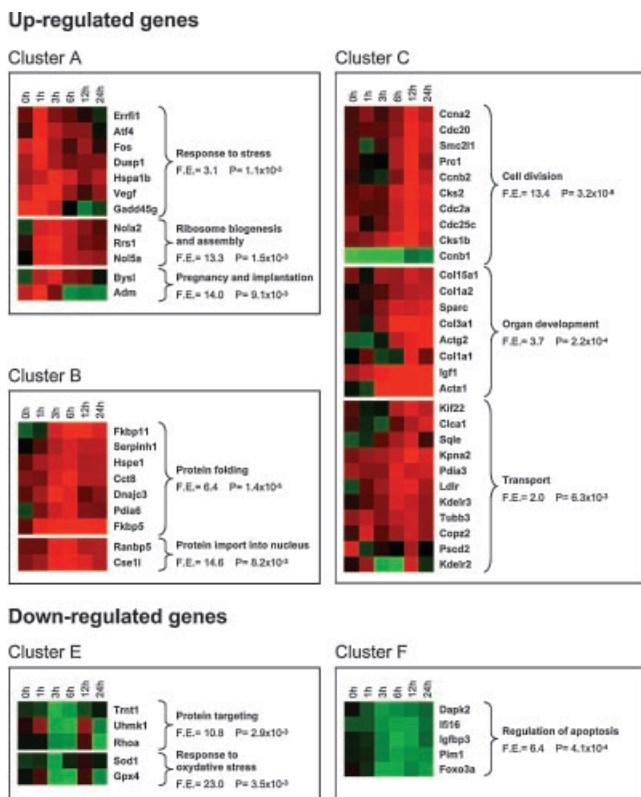


Figure 3. Relevant list of up- and down-regulated genes in each cluster. Relevant gene names in each cluster are indicated. Changes in expression levels are also indicated by color based on normalized values (green, black and red for low, intermediate and high values, respectively) with the time points (in hours) indicated above. Major subclasses of gene functions with their corresponding Fold Enrichment (FE) and P-value (P) are also displayed for each cluster.

DHT Modulates Genes Involved in the ATM/Gadd45g Signaling

As our GeneChip analyses highlighted some genes related to the ATM/Gadd45g signaling and in order to confirm the temporal changes of gene expression levels of major genes involved in cell cycle regulation, we performed QRT-PCR, as illustrated in Figure 4. Briefly, 17 genes involved in

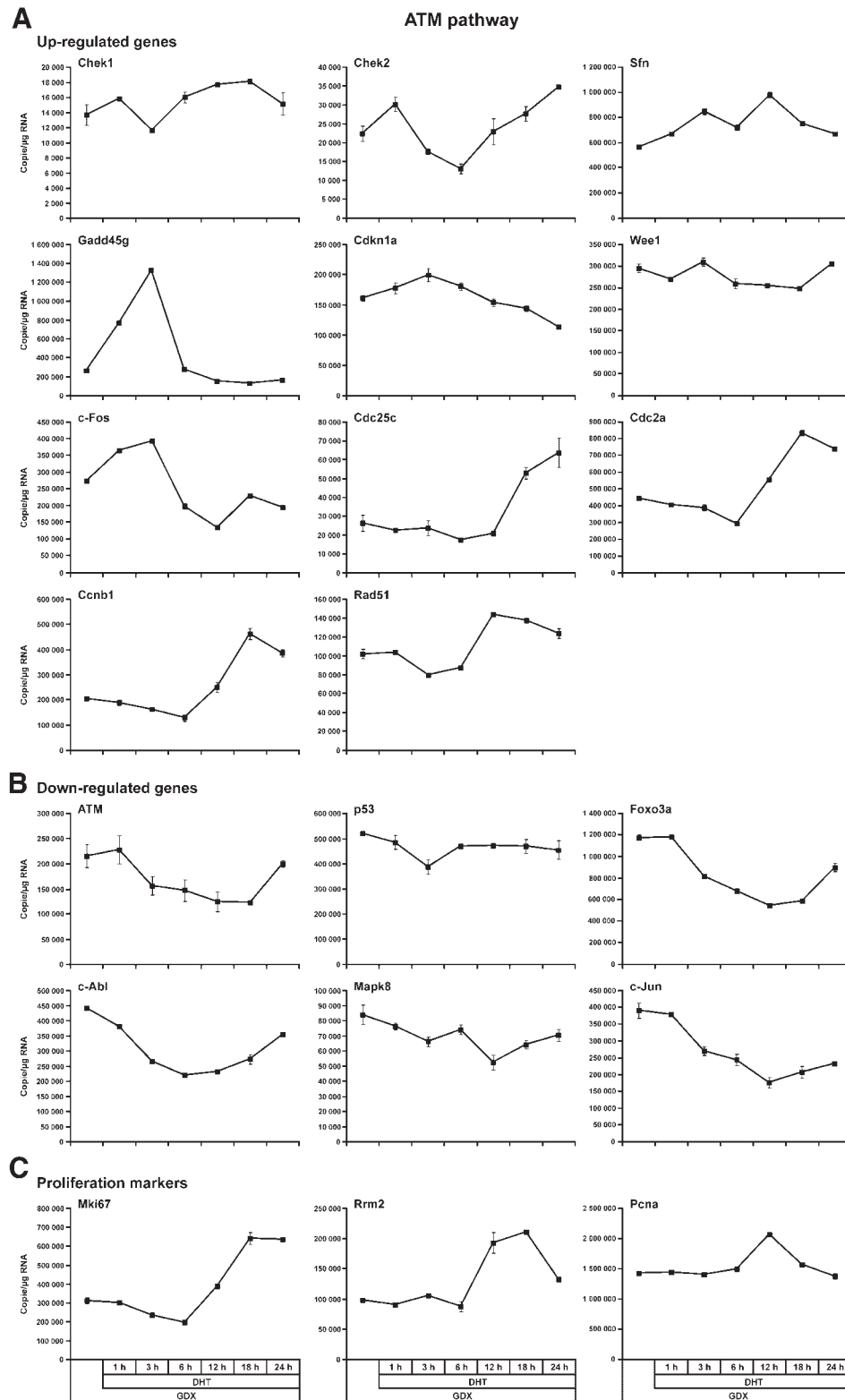


Figure 4. Temporal changes of gene expression levels performed by QRT-PCR. Evidence for transcriptional regulatory network during the uterotrophic response of ATM signaling-related genes and selected genes involved in the G2/M checkpoint. Panel A: up-regulated genes. Panel B: down-regulated genes. Panel C: selected proliferation markers. The number of copies by μg /RNA is indicated on the Y-axis and the time points on the X-axis.

the ATM and Gadd45g signaling pathway were analyzed by QRT-PCR as well as three proliferation marker genes, namely Ki67, Rrm2 and pCNA. The expression of Ki67 and Rrm2 is up-regulated at 18 hr, to an extent of 2.0- and 2.2-fold, respectively, while pCNA is stimulated of about 1.5-fold at 12 hr. All genes tested displayed a 95% correlation rate between microarrays and QRT-PCR if we consider the sense of the regulation (up- or down-regulated). The global correlation analysis using the coefficient of Pearson and Spearman revealed a correlation of 0.715 and 0.675, respectively (data not shown) between Genechips hybridization and QRT-PCR analysis. Indeed, only *Wee1* gene identified with a 3.2-fold stimulation by GeneChip analysis, demonstrated no appreciable regulation using QRT-PCR.

At first, *Gadd45g*, which can be transcriptionally activated by p53, is clearly and strongly induced within 3 hr. However, no clear stimulation of ATM or p53 can explain this up-regulation of *Gadd45g*, both genes being even slightly down-regulated. Moreover, given that the stimulation of other genes known to repress the cell cycle like Chk1, Chk2, Cdkn1a and *Wee1* displayed a weak up-regulation, a trend towards a repression of the cell cycle at the G2/M checkpoint in the early phase can be observed. However, in the late phase of DHT stimulation (18–24 hr), a significant up-regulation of genes involved in the G2/M transition is observed. Indeed, both *Cdc2a* and *Ccnb1* genes are up-regulated within 18 hr. Then, the *Cdc25c* gene is gradually and strongly induced within 24 hr.

Other genes like *c-Fos* and *Sfn* are about 2-fold (2.6- and 2.1-fold, respectively, in GeneChip analysis) up-regulated at 3 hr and 12 hr, respectively. The *Foxo3a* gene, involved in

the repression of p53, is down-regulated at 12 hr but since p53 does not display any up-regulation, the role of *Foxo3a* regarding the p53-dependent regulation appears unclear.

On the other hand, the *c-Abl* and *c-Jun* oncogenes are markedly (>2-fold) down-regulated within 6 hr and 12 hr, respectively, while *Mapk8* and *Rad51* are slightly down- and up-regulated, respectively, during this phase.

Taken together, as presented in Figure 5, these data suggest that DHT early induces the transcriptional activation of *Gadd45g*, which plays a critical role in G2/M arrest and induction of apoptosis signaling. On the other hand, DHT lately promotes the inhibition of genes of the ATM signaling involved in the G1/S checkpoint and triggers transcriptional increases of *Rad51*, which plays an important role in double-strand break recombination repair, as well as genes whose products are crucial for the G2/M transition and cell cycle progression.

DISCUSSION

In the present study, ovariectomized mice were exposed to DHT for up to 24 hr. According to our data, there is evidence of rapid induction of transcriptional regulator genes and signaling components followed by the late stimulation of cytoarchitectural genes by DHT. Evidence from our previous analyses (data not shown) demonstrated a fivefold increase of uterine weight following a prolonged DHT treatment (3 weeks or more) compared to a eightfold increase when OVX mice are treated with E2 (Ivanga et al., 2006). Similar DHT effects on uterine weight have also been reported

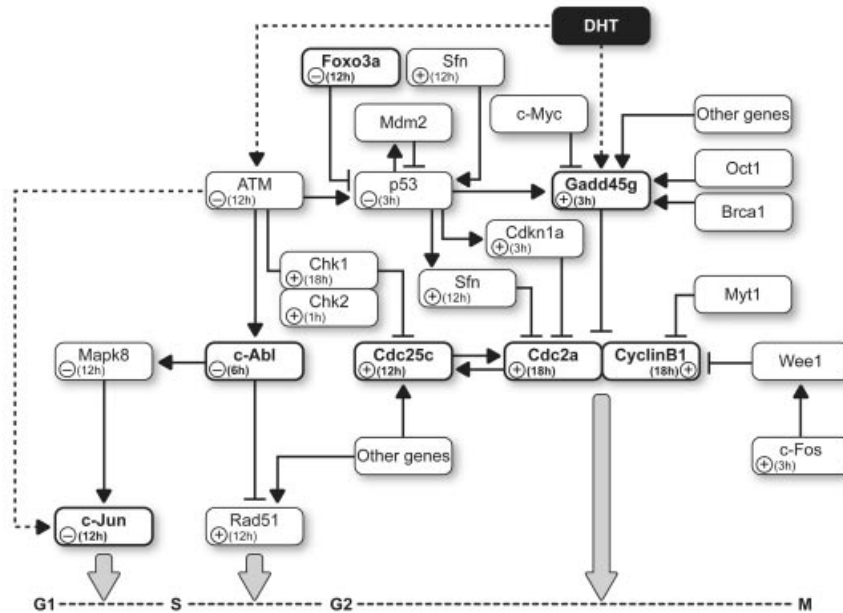


Figure 5. Schematic and simplified representation of the ATM/Gadd45g transcriptional cascade triggered by DHT, in the mouse uterus. DHT activates a transcriptional cascade resulting in the modulation of genes involved in the cellular checkpoints control. All key genes have been confirmed by QRT-PCR and their DHT-modulation, stimulation or repression, is indicated by + or -, respectively, while

genes demonstrating a significant regulation (more than twofold) are highlighted in bold. Time points of the modulations of analyzed genes by QRT-PCR are also indicated. Other genes include known and unknown upstream targets of the corresponding genes while dashed line suggests a direct or indirect regulation.

following a single treatment of 24 hr (Nantermet et al., 2005). These presented results are in agreement with the estrogen-like effect of androgens, further suggesting that a 0.1 mg dose of DHT can induce, in 24 hr, the promotion of the cell cycle in mice. It is likely that DHT regulates the trophic environment and architecture of the rodent uterus through a gene expression program, which is overlapping but distinct from the estrogen response, as well the magnitude of these DHT-induced changes appear to be less important than those caused by the action of E2 (Nantermet et al., 2005).

During the first phase (from 0 hr to 12 hr), the data indicate that DHT induces chaperonins (Hsp105, Hspa1a and 1b) and ribosomal proteins expression. Indeed, some chaperonins would participate in cell proliferation by interacting with proteins needed for the proliferation process (Parcellier et al., 2003), and it has been shown that decreasing Hsp70 expression levels in tumor cells could induce apoptosis and cell growth inhibition (Wei et al., 1995; Kaur et al., 2000). Androgens are also recognized to stimulate ribosomal RNA synthesis in LNCaP cells and rat prostate (Kabler et al., 1996). Moreover, at this step, DHT also up-regulates transcription factor genes such as *Aff4*, whose product in human osteoblasts has been found to post-transcriptionally regulate the synthesis of Type I collagen (Yang et al., 2004), which is a constituent of the extracellular matrix of uterus, being stimulated in a late window of treatment. Among the growth/apoptosis-related genes early-modulated by DHT, most oncogenes are induced, while others, such as c-Jun, are repressed. It has to be stated that proto-oncoproteins are involved in the transcription of downstream proteins, and deregulation of their expression can result in oncogenic transformation or inhibition of apoptosis (Tsatsanis and Spandidos, 2004).

Furthermore, some stress and immunity-related genes (e.g. *Gadd45g*) are increased during this first phase. Interestingly, DHT has been shown to have proatherogenic effects on vascular cells, and to induce proinflammatory events in male endothelial cells (Death et al., 2004). However, other immunity-related genes and especially myeloid cell differentiation-related genes, such as *Foxo3a* and *Iff16*, appear to be down-regulated by DHT. Particularly T-cell and B-cell functions have been demonstrated to be decreased by androgens (Ahmed et al., 1987; Slater and Schreck, 1993).

During the second phase of the process (from 12 hr to 24 hr), and consistent with the estrogen-like effect, DHT stimulates the expression of cytoarchitectural (tissue and organ development) and lipid (sterol metabolism and biosynthesis)-related genes. Indeed, the extracellular matrix-cell adhesion molecules genes (e.g. Type I procollagens) and the structural and cytoskeleton factors genes (e.g. keratin complexes) are principally lately increased by DHT, along with most lipid transport and metabolism-related genes. These results are in agreement with DHT-associated cell proliferation effects previously reported (Armstrong and Papkoff, 1976; Zhang et al., 2004). Among the cell cycle-related genes, DHT induces several cyclins, such as the cyclinA2 and B1, which are involved in the G1/S and G2/M transition, respectively. Repression of both *Igfbp3* and *Foxo3a* genes is also con-

sistent with the fact that their repression normally allows the progression of the cell cycle (Hung and Gibbons, 1983; Tuckerman et al., 2000).

Comparison of androgen-regulated genes analyzed in the current study with the only similar investigation (to our knowledge) reported to date (Nantermet et al., 2005), highlights the pitfalls of using a single DHT treatment of 24 hr instead of several time points as performed in the present study. Using a single time point, it is indeed impossible to detect the fine regulation of genes involved in cell cycle control or other functions required for uterine biological processes. One striking example is illustrated by the regulation of the *Adm* gene, which was reported as down-regulated by androgens after 24 hr (Nantermet et al., 2005). However, when using multiple time points, we observe a stimulation of this gene of about 3.7-fold at 3 hr. Several other regulated genes were not detected over a 24 hr period, namely *Mcnd* and *Mcnd5* involved in cell cycle control, *Fkbp11* and *Fkbp5* (chaperone genes), *VEGF*, the transcription factors *Fos* and *Wnt4*, and the *Ramp3* gene involved in glycosylation which are all up-regulated within 24 hr.

In addition, growth factors, like *Igf1* and *Igfbp3* seem more strongly affected over a short-time period (12 hr) than the mRNA expression change observed at 24 hr. Moreover the signal transduction component *Dusp1* is up-regulated by 2.3-fold at 3 hr, while a down-regulation of 1.6-fold was reported at 24 hr (Nantermet et al., 2005). This illustrates the importance to analyze the fine regulation of the cell cycle components since events of cell cycle progression are taking place over a short time period. Besides, if we consider the major ATM/Gadd45g genes pathway modulated by DHT (>2-fold) in this study, namely *Gadd45g*, *c-Jun*, *c-Abl*, *Foxo3a*, *Cdc25c*, *Cdc2a* and *Ccnb1*, none has been reported as regulated in the investigation previously performed (Nantermet et al., 2005).

As described previously (Hewitt et al., 2003; Moggs et al., 2004; Ivanga et al., 2006), it is of interest to mention that E2 has a stronger effect of stimulation on IGF1 pathway than that observed with DHT treatment. It has been reported that *IGF1*, a known androgen-responsive gene, is not induced by DHT in absence of estrogen-receptor alpha (ER α), supporting that this receptor is needed to mediate DHT action (Nantermet et al., 2005). Accordingly, in ER α knockout (ERKO) mice, no estrogen-responsive genes other than AR are modulated by DHT, while in presence of ER α , when using a combination of DHT with an anti-androgen, *IGF1* mRNA expression is not induced in prostate (Nantermet et al., 2005), supporting the role of AR in this regulation.

Given that both AR and progesterone receptor (PR) bind to identical consensus hormone response elements (HREs), it is also of interest to compare P- and DHT-regulated genes. However, comparison of DHT-regulated genes with the most exhaustive study describing gene expression induced by P (Jeong et al., 2005) identified only few genes modulated by both hormones. This apparent gene expression difference could be explained in part by a recently identified new class of response elements, which are preferentially stimulated by AR (Sommer and Haendler, 2003; Verrijdt et al., 2003; Geserick et al., 2005). Altogether, it appears that gene expression following DHT treatment is more similar to E2

gene regulation than effects observed following a progesterone treatment.

To further support our observations involving DHT in the ATM-mediated regulation of the cell cycle, we have developed a scheme illustrating how these genes interact and affect one another (Fig. 5), and all these gene regulations were further confirmed using QRT-PCR (Fig. 4).

ATM, involved in DNA damage response signal transduction resulting in induction of apoptosis or growth arrest, interacts with c-Abl (Baskaran et al., 1997) and may drive its down-regulation within 12 hr. The down-regulation of c-Jun (within 12 hr and 18 hr), which has been demonstrated to be a downstream target of c-Abl tyrosine kinase (Barila et al., 2000), could favor the G1/S phase progression (Schreiber et al., 1999). However, c-Jun has also been associated with apoptosis due to its homeostatic function, which is dependent of the cell environment (Shaulian and Karin, 2001). It should be noted that c-Jun is also a direct downstream target of ATM (Foray et al., 2003) and is a negative regulator of p53 expression (Schreiber et al., 1999; Shaulian and Karin, 2001).

On the other hand, Rad51 mRNA expression, which is stimulated by down-regulation of c-Abl (Yuan et al., 1998), displays a slight stimulation (18 hr). In this case, since c-Abl, given its late and slight down-regulation, does not seem to explain the activation of *Rad51*, it appears that other kinases could be involved in this stimulation.

ATM also interacts with the tumor suppressor p53 (Kurz and Lees-Miller, 2004), which is known to transcriptionally activate *Gadd45g*. Indeed, later, *i.e.* from 12 to 24 hr, the expression of the *Gadd45g* gene is low. Consequently, at this late step, our results show that the genes *CyclinB1* and *Cdc2a*, whose complex activity is repressed by *Gadd45g*, are indirectly induced by DHT. Moreover, the Cdc2/CyclinB kinase complex activity is crucial for G2/M transition (Sancar et al., 2004). More important, Cdc2 is activated by Cdc25c (Lee et al., 1992), which is consistently induced by DHT from 12 to 24 hr. Briefly, these results suggest that the G2/M arrest is abrogated during the second phase of regulation, enabling the stimulation of the cell cycle. Moreover, DHT strongly and quickly induced (within 3 hr) *Gadd45g* gene, whose product is known to play pivotal roles in G2/M arrest and induction of apoptosis signaling, through the dissociation of the cyclinB/Cdc2 complex (Lee et al., 1992; Taylor and Stark, 2001). In agreement with these results, previous observations have shown that *Gadd45g* is an up-regulated androgen-responsive gene with growth inhibitory activity in human prostate cancer cells showing a rapid and transient dose-dependent stimulation followed by a decreased expression after 48 hr (Jiang and Wang, 2004). However, these results support that this up-regulation is sensitive to protein synthesis inhibition suggesting an indirect effect of androgen on *Gadd45g* promoter. Besides, no androgen response element (ARE) could be identified in the proximal promoter of *Gadd45g*. Therefore, since no significant induction of ATM and p53 mRNA expression could be observed which could explain the modulation of downstream targets like c-Abl and *Gadd45g*, it is likely that *Gadd45g* gene is up-regulated by androgen in an ATM-independent manner. Upstream activators of *Gadd45g* remain to be identified

since other activators like Brca1, NF-YA and Oct1 do not display any suggestive modulation.

In this study, we have shown that physiological level of DHT causes, within 24 hr, the modulation of genes likely involved in ATM-independent pathways, as well as the transcriptional modulation of genes whose products can promote the cell cycle progression in mice. It is reasonable to assume that, in the mouse, DHT could trigger transcriptional cascades, which regulate uterine cell growth, probably via a tightly concentration-dependent control of the G1/S and G2/M cellular checkpoints. Thus, this mechanism may be of importance in the cyclical changes of uterine cells in the mouse uterus, in which androgens seem to play a crucial role in the process.

METHODS

Animals and Treatment

Ten- to eleven-week-old female C57BL/6 mice were received from Charles River (St. Constant, Canada) and were allowed to acclimate for 4 weeks. The animals were housed individually in an environmentally controlled room (temperature: $22 \pm 3^\circ\text{C}$; humidity: $50 \pm 20\%$; 12-hr light–12-hr dark cycles, lights on at 07:15 hr). The mice had free access to tap water and a certified rodent feed (Lab Diet 5002 (pellet), Ralston Purina, St. Louis, MO). The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals.

Animals, weighing 20–27 g (mean 23.3 g), were randomized according to their body weights and were assigned to 7 groups of 14 animals each as followed: (1) Ovariectomized (OVX) control; (2–7) OVX + DHT (0.1 mg/mouse). On day 1 of the study, animals were bilaterally ovariectomized under isoflurane anesthesia. Prior to the necropsy performed on day 8 of the study, mice received a single subcutaneous injection (0.2 ml/mouse) of the vehicle alone (5% ethanol–0.4% methylcellulose; group 1) or DHT (groups 2–7). The injection of vehicle was performed 24 hr prior to necropsy for animals into group 1 while DHT was injected 1 hr (group 2), 3 hr (group 3), 6 hr (group 4), 12 hr (group 5), 18 hr (group 6) or 24 hr (group 7) prior to necropsy.

On day 8 of the study, mice under isoflurane anesthesia were exsanguinated at the abdominal aorta followed by cervical dislocation. The uterus was collected and rapidly frozen in liquid nitrogen. For each group, the 14 uteri collected were pooled and tissues were kept at -80°C until RNA extraction.

RNA Isolation and Microarray Hybridization and Analysis

Total RNA was isolated using Trizol (Invitrogen, Burlington, Ontario) following manufacturer's protocol and cDNA synthesis was performed as previously described (Ivanga et al., 2006). cDNA was *in vitro* transcribed using a T7 BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) to produce biotinylated cRNA. cRNA purification and fragmentation were performed as described previously (Ivanga et al., 2006).

cRNA probes were hybridized to MG-U74v2 GeneChip Set (Affymetrix, Santa Clara, CA) and subsequent steps of hybridization were performed as reported previously (Ivanga et al., 2006). cRNA probe from the control group was hybridized on two microarrays (duplicate) while cRNA probe corresponding to each time point was hybridized on separate microarrays. Microarrays staining and scanning were then processed as previously described (Ivanga et al., 2006). Signal intensities for β -actin and *GAPDH* genes were used as internal quality controls. The ratio of

fluorescent intensities for 5' and 3' of these housekeeping genes was <2. Scanned images were analyzed and normalized (target intensity equal to 500) with Microarray Suite 5.0 (Affymetrix).

Differential expressed genes were selected using a variable limit fold change (LFC) (Mutch et al., 2002) decreasing with gene expression value ($LFC = a + (b/x)$ where x is the minimum intensity of gene expression). The curve was estimated based on a ratio distribution calculated from replicated chips. The resulting cut-off point, $LFC = 1.9 + (60.0/x)$, produces an approximately constant rate of false positive modulated genes of 0.1%. Genes over these criteria must also be called present in at least two points of the experiment using MAS5 program. Affymetrix MAS5.0 normalized signal intensities were submitted to GEO (<http://www.ncbi.nlm.nih.gov/geo/>) and are available under the following series ID: GSE6237.

Clustering Analysis

The differential expressed genes were clustered using the GeneCluster2 software based on a self-organizing map algorithm. Genes showing a max/min ratio greater than 1.9-fold in their gene expression levels and above 300 in their expression intensities were selected. Selected genes were then normalized to obtain a mean value of zero and a standard deviation of one, and the normalized data were thereafter clustered. The L2L software was used to classify genes from each cluster into biological processes (Newman and Weiner, 2005). When the genes were not classified by this software, EASE software (Hosack et al., 2003) was used to identify the biological process associated with the gene. Using L2L, the Fold Enrichment (FE) of specific subclasses of gene functions was also calculated (representing the ratio of the number of regulated genes observed in the cluster/expected number of genes belonging to this subclass found on the GeneChip) along with the corresponding P -value derived from a binomial distribution (representing the statistical significance of the overlap between both lists of genes: observed vs. expected). The genes were classified in more than one major biological process (when applicable) since several proteins can demonstrate many different physiological activities in the cell metabolism, which is more representative of the gene function.

Quantitative Real-Time PCR (QRT-PCR)

Total RNA was purified using RNeasy MinElute Cleanup kit (QIAGEN, Valencia, CA) after a digestion step with DNase I (QIAGEN, Valencia, CA). The quality of RNA was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

First-strand cDNA synthesis was accomplished using 5 μ g of isolated RNA as reported previously (Ivanga et al., 2006). The resulting products were purified with Qiaquick PCR purification kits (QIAGEN, Valencia, CA). cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler Realtime PCR apparatus (Roche Inc., Nutley, NJ). Reagents were obtained from the same company and used as described by the manufacturer. PCR amplification and reading of the fluorescence signal was performed as previously described (Luu-The et al., 2005; Ivanga et al., 2006). Each sample was performed in duplicate. Data calculation and normalization was performed using second derivative and double correction method as described previously (Luu-The et al., 2005) and using the housekeeping gene hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1). Hprt1 gene is recognized to display stable expression levels from embryonic life through adulthood in various tissues (Warrington et al., 2000). mRNA expression levels are expressed as number of copies/ μ g total RNA using a standard curve of Cp versus logarithm of the quantity. The standard curve was established using known cDNA amount of 10 , 10^2 , 10^3 , 10^4 , 10^5 and 10^6 copies of Hprt1 and a Light Cycler 3.5 program provided by the manufacturer (Roche Inc.).

ACKNOWLEDGMENTS

We would like to thank all the research assistants involved in animal care and treatments for their skillful technical assistance. A special thank to Dr. Van Luu-The and his group, particularly to Nathalie Paquet, for QRT-PCR quantification. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. This work was supported by Genome Canada and Genome Québec for the ATLAS project. M.I. is a recipient of a CIHR (Canadian Institutes of Health Research) studentship as part of a training program in genomics ("Génomique fonctionnelle des maladies endocriniennes") and of a studentship of the gabonese government. F.D. is a recipient of a chercheur boursier from the Fonds de la Recherche en Santé du Québec (FRSQ) and a Research Career Award in the Health Sciences from CIHR/Rx&D Health Research Foundation.

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