Gene and protein expression in the myometrium in pregnancy and labor

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Abstract

Microarray technologies widen our comprehension of the major structural and metabolic transformations which affect the myometrium from the very beginning of pregnancy until parturition. The results are coherent with the mass of information which was accumulated previously, primarily on the basis of studies of selected critical factors. They highlight the activation of precise signaling pathways, some of which may have been previously under evaluated. The remodelling and maturation processes that the myometrium undergoes in pregnancy appear clearly as phenomena which last during the full course of gestation. Comparatively, the onset of labor is perhaps the phenomenon which remains the least well described by these methods of analysis. Nevertheless, genomic studies constitute a necessary first step of orientation and help establishing new links between the generic signaling pathways that are activated during the normal or pathological gestation. These studies also represent an indicative step that will have to be paralleled, in the future, with the results of the systematic proteomic analysis of the myometrium.

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Introduction

Parturition encompasses composite physiological processes that require synchronization of uterine contractions, cervical dilatation and fetal membrane rupture. The mechanisms by which parturition is triggered remain unknown, but they as are all developmental processes, the results of timely coordinated biochemical and physiological steps. Over the past decades, reductionist approaches have investigated the cellular and molecular bases of these developmental changes and focused on assessing the regulation of selected critical factors involved in the onset of labor.

Recently, the near-entire genomic sequence of the human and several model animals have provided the opportunity to enhance our understanding of the relationships between genes, phenotypes and global transcriptional status. Oligonucleotides or DNA microarray technologies allow the examination of the function of thousands of genes at once and in parallel, thereby providing an 'assay' of the transcriptional status of cells or tissues in a wide variety of physiological or pathophysiological situations (Dunckley *et al.* 2005). In the context of uncomplicated or complicated pregnancy and parturition, their interest is to obtain a molecular

snapshot of the expression profile of gene transcripts as a function of the time-dependent process regulating myometrial activity.

Functional genomic studies during pregnancy

Functional genomics has arrived at the study of parturition in different species: mice (Bethin *et al.* 2003, Salomonis *et al.* 2005); rats (Girotti & Zingg 2003) and humans (Chan *et al.* 2002, Marvin *et al.* 2002a, 2002b, Tashima *et al.* 2002, Bethin *et al.* 2003, Charpigny *et al.* 2003, Keelan *et al.* 2003, Bailey *et al.* 2005, Esplin *et al.* 2005, Havelock *et al.* 2005). Also in different tissues: myometrium (Aguan *et al.* 2000, Chan *et al.* 2002, Bethin *et al.* 2003, Charpigny *et al.* 2003, Esplin *et al.* 2005, Havelock *et al.* 2005, Salomonis *et al.* 2005); primary cultures of human myometrial cells (Bailey *et al.* 2005); fetal membranes (Muhle *et al.* 2001, Chen *et al.* 2002, Marvin *et al.* 2002a, 2002b, Tashima *et al.* 2002, Keelan *et al.* 2003) and trophoblasts (Chen *et al.* 2002, Kato *et al.* 2002). Various methodologies have been used: suppression subtractive hybridization (SSH) (Chan *et al.* 2002) or microarrays for most of the previous cited studies. All investigators have reported genes differentially regulated

during parturition (Romero *et al.* 2002) and multiple novel candidate markers for preterm labor (Wu *et al.* 1999, Chan *et al.* 2002, Marvin *et al.* 2002a, 2002b). They contributed to shed light on specific pathophysiological issues such as the patterns of expression of cytokines in the fetal membranes and decidua particularly in the presence of intrauterine infection (Keelan *et al.* 2003) or on the induction of enzymes for prostaglandin synthesis (Bethin *et al.* 2003) which are commonly associated with the onset of labor. Groups of genes with as yet unknown functional connection to these pathologies were also found coordinately expressed. This was emphasized in animal studies which allow the exploration of global gene expression patterns and their co-regulation on the basis of their genomic location over the full time-course of myometrial transformation (i.e. pregnancy), following experimentally-controlled infection (Wu *et al.* 1999, Muhle *et al.* 2001, Girotti & Zingg 2003, Salomonis *et al.* 2005) or in human studies following transfection of cultured myometrial cells with CREB, CREMalpha and CREMtau2alpha cDNAs, to affect the expression of genes (Bailey *et al.* 2005).

Computational methods have significantly helped to the interpretation of gene profiling experiments by delineating clusters of genes sharing coherent expression features (Claverie 1999). During the last five years, statistical methods and data analysis for array studies have progressed enormously and today an 'ideal' study should now incorporate i) careful research designs (differential expression or cross sectional studies), ii) statistical methods incorporating background adjustment and normalization of data, assessment of differential expression after determining sample size to control the proportion of positive calls that are false positives (false discovery rate), iii) hierarchical clustering (e.g. HOPACH method; Ganesh *et al.* 2004, Salomonis *et al.* 2005), iv) functional organization of genes into pathways or networks with the aid of integrated databases like the gene ontology consortium (Lewis 2005) or the Kyoto encyclopedia of genes and genomes (KEGG; Kanehisa 1997, Kanehisa & Goto 2000) and finally v) integrating genotype, transcription and clinical trait data (Salomonis *et al.* 2005).

The past studies on the human myometrium have reported on different microarrays which contained different sets of genes/expressed sequence tags (ESTs) and each study has profiled a small number of patients due to various constraints. More gene expression profiling information would be a welcome addition to our knowledge base of parturition. The present review examines transcriptional differences between the preterm quiescent myometrium, term myometrium not in labor and term myometrium in labor. Attention is focused on genes expressed essentially in the 'normal' human myometrium. From identified genes that were differentially expressed, we identified biological pathways based on these genes. One study has reported a direct comparison of the non-pregnant myometrium (NP) with the pregnant human myometrium at term not in labor (TNIL; Rehman *et al.*

2003). It gives an overall picture of the changes that the uterine muscle undergoes in its adaptation to gestation. Two studies have described the changes in gene expression in the myometrium in preterm patients not in labor (PTNIL) versus patients at TNIL (Bethin *et al.* 2003, Charpigny *et al.* 2003) and two additional studies have described the changes in gene expression in the myometrium in patients at TNIL versus patients at term in labor (TIL) (Esplin *et al.* 2005, Havelock *et al.* 2005). Other studies could not be compiled directly, either because they did not provide sufficient information on the genes being studied (i.e. lack of GenBank or Unigene references), they considered other components of the utero-fetal unit (i.e. membranes, decidua, cervix, etc.) or they were carried out on animal species.

To translate lists of tens or hundreds of genes found to be differentially regulated in the conditions under study into a clearer understanding of the biological phenomena involved, we used combinations of searches through the literature referenced in public databases and the Onto-Tools software developed by the Draghici's group at Wayne State University, (Detroit, MI, USA; <http://vortex.cs.wayne.edu/Projects.html>). The Onto-Express module helps to recognize functional profiles (using gene ontology terms) for the categories: biochemical function; biological process; cellular role; cellular component; molecular function, and chromosome location (Draghici *et al.* 2003). The Pathway-Express module helps data mining – proposing on the basis of a computational method a hierarchical list of several KEGG pathways (Kanehisa 1997, Kanehisa & Goto 2000) most likely adjusted to changes observed in microarray experiments (Khatri *et al.* 2005). KEGG is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information (<http://www.genome.jp/kegg/pathway.html>). Because relevant raw data for myometrium microarrays were not always available, input data files for use with the Onto-Tools software modules were built on gene lists reported in published papers or, when available, on the complete gene expression data set, deposited as supplemental data at a public Internet site, see Bethin *et al.* 2003, Charpigny *et al.* 2003, Rehman *et al.* 2003. Drawbacks and limitations inherent to the use of Onto-Tools or of their cognate alternatives for ontological analysis, have been reviewed (Khatri & Draghici 2005). These limitations remain to the present day, questions over the robustness of array data and the criteria under which their conclusions were drawn have been made. How can one compare the reproducibility of data on two or more different array platforms or between laboratories using the same or different array platforms? Are there groups of genes that one can almost use as quality controllers for gestational changes? The recent review by Allison *et al.* (2006) has the merit not of providing definitive answers on these questions but of proposing simple sound recommendations for future microarrays analysis methods.

In the pregnant human myometrium, the differential expression of 118 genes could be dispatched in 14 main KEGG pathways that are the most representative (see Khatri *et al.* 2005, for the rationale of KEGG pathway search) of the changes seen in NP versus TNIL, PTNIL versus TNIL, and TNIL versus TIL. However, other genes not identified here may be involved in known pathways with importance in myometrial functions, because the KEGG itself is permanently evolving, the functions of many genes are yet to be defined under various contexts and arrays used in the referred studies may not have contained all known genes listed in the KEGG base. Tables 1–4 and Figs 1–2 summarize the compiled data which are commented on below in more detail. Because our discussion is also largely drawn from literature reports of relevant protein changes in human and other animal species, as well as in model cell culture systems, mention of the species or model systems is made from which we quoted this supplementary data. Wherever possible, OMIM nomenclature has been adopted in the text and tables (<http://www.ncbi.nlm.nih.gov/omim/>).

Changes in structural and contractile genes during gestation

Actin cytoskeleton, focal adhesion, adherens and tight junction related genes represent a large subset of genes that are over-expressed in the pregnant human myometrium as compared to the non-pregnant state (Table 1).

Regulation of actin cytoskeleton

Growth and cytoskeletal remodeling of myometrial cells during pregnancy are critical for myometrial functions including those expressed during labor and delivery. At term, signals that initiate labor, ultimately promote a switch in the phenotype of the quiescent uterus to a smooth muscle which becomes spontaneously active, excitable, highly responsive to uterine agonists, and exhibits a high degree of cell-cell coupling. Myometrial cells are rich in actin microfilaments, intermediate filaments and microtubules that allow cells to adapt to a variety of shapes and to carry out coordinated and directed movements. In rat, myometrial expression of alpha-actin is high throughout pregnancy. An increased expression of gamma-actin and its translocation to the membrane is observed in uterine myocytes at late gestation. Therefore, the alteration in myometrial composition of contractile proteins is important to prepare the myometrium for the development of contractions during labor (Shynlova *et al.* 2005).

RhoA, a member of the Ras superfamily, and its downstream mediator RhoA kinase (ROCK1) are necessary for agonist-induced stress fiber formation in human myometrial cells (Gogarten *et al.* 2001). The higher expression of protein kinase C (PKC) isoforms observed in pregnant *versus* non-pregnant myometrium (Rehman *et al.* 2003) may promote or drive the formation of stress fibers.

Additionally, up-regulation of ROCK1 has been demonstrated in human term myometrium (Moore *et al.* 2000, Rehman *et al.* 2003). This leads to the idea that increased endogenous ROCK1 activity, resulting in enhanced RhoA-mediated calcium sensitization, is involved in the increased contractility at the time of labor. It is of note that epidermal growth factor (EGF) increases the presence of actin and myometrial EGF receptor transcripts are increased at the time of parturition in humans (Gargiulo *et al.* 1997, Charpigny *et al.* 2003, Rehman *et al.* 2003).

Focal adhesion, adherens junctions and tight junctions

The transmission of force between the contractile apparatus of the cell and extracellular matrix (ECM) occurs at membrane-associated dense plaques or 'focal adhesions'. The homeostasis of ECM relies upon the intricate interactions between collagens (ECM components), integrins and the associated signaling molecules (focal adhesion molecules). This in turn regulates adherens and tight junction dynamics. Focal adhesions consist of clusters of integrins that mediate interactions between the extra- and intra-cellular environments. The cytoplasmic regions of integrins connect with cytoskeletal elements and signaling components such as focal adhesion kinase (FAK), while the extracellular regions connect to specific extracellular matrix molecules such as collagen, laminin or fibronectin.

Fetal growth imposes mechanical tension on the myometrium at term, which in turn induces activation of FAK leading to focal adhesion turnover and supporting myometrial cell hypertrophy. During late pregnancy, a fall in tyrosine phosphorylation of FAK and stabilization of focal adhesions occur for provoking the cessation of myometrial hypertrophy. Because actin polymerization and the dynamic remodeling of the actin cytoskeleton play key roles in the regulation of myometrial contraction, there is growing evidence that stretching induces labor, probably through a change in the expression of contraction associated proteins (CAPs) as observed in rat myometrium (Macphee & Lye 2000). In human term pregnant myometrium, changes in cytoskeletal organization support a role for FAK and other focal adhesion-associated proteins as regulators of actin dynamics. Fibronectin receptor-alpha subunit (ITGA5) with its known partner ITGB1, is up-regulated in rat myometrium during late pregnancy and labor (Williams *et al.* 2005) as well as vitronectin receptor-alpha polypeptide (ITGAV) in the TNIL human myometrium (Rehman *et al.* 2003). Therefore they may interact with actin-binding proteins (e.g. actinin and filamin) to form mechanical links to the cytoskeleton. A strong expression of vinculin is concentrated at actin–vinculin focal adhesion sites in human myometrial cells (Yu & Lopez Bernal 1998). At the time of parturition, expression of integrins declines and the focal adhesion and related pathways do not change in studies which compare the TNIL and TIL states of pregnancy (see Table 1).

Table 1 The main KEGG metabolic pathways, regulatory pathways or molecular complexes that were found to be expressed in the myometrium on the basis of genes detected in genomic studies according to ¹NP–TNIL, to ²PTNIL–TNIL or to ³TNIL–TIL, during the cycle or pregnancy. All gene transcripts showed at least a 2 fold or more, up (+) or down-regulation (–) after paired comparisons of the following stages: non-pregnant (NP) versus term not in labor (TNIL), preterm (PTNIL) versus TNIL, or TNIL versus term in labor (TIL). Brackets: a number of genes represented in a defined pathway. A total of 118 genes were sorted as belonging to any of the 14 pathways, 0: no gene represented for the corresponding pathway. OMIM nomenclature adopted for gene names (see text).

Actin cytoskeleton			Focal adhesion			Adherenes junctions			Tight junctions		
NP–TNIL ¹ (15)	PTNIL–TNIL ² (6)	TNIL–TIL ³ (0)	NP–TNIL ¹ (18)	PTNIL–TNIL ² (6)	TNIL–TIL ³ (3)	NP–TNIL ³ (11)	PTNIL–TNIL ² (3)	TNIL–TIL ³ (0)	NP–TNIL ¹ (10)	PTNIL–TNIL ² (0)	TNIL–TIL ³ (0)
ACTN4(+) CD14(+) EGFR(+) FGFR1(+) HEM1(+) ITGAM(+) ITGAV(+) ITGB5(+) PFN1(+) PIK3CA(+) PIP5K1C(+) RAC2(+) ROCK1(+) RRAS(+) VCL(+)	EGF(–) ITGAV(–) ITGB3(–) ITGB6(–) MATK(–) PIK3CB(–)		ACTN4(+) ARHGAP5(+) CAV2(+) COL1A2(+) EGFR(+) FLNA(+) ITGB5(+) PIK3CA(+) PIP5K1C(+) PRKCB1(+) PRKCG(+) ROCK1(+) RRAS(+) TNC(+) VASP(+) VCL(+) VWF(+) ZYX(+)	INSR(+) ITGB3(–) ITGB6(–) MAPK10(–) PIK3CB(–) RAP1A(–)	ILK(+) THBS1(+) TNC(+)	ACTN4(+) CTNNA1(+) EGFR(+) FGFR1(+) NLK(+) PTPN6(+) PTPRJ(+) RAC2(+) SMAD2(+) TCF7L2(+) VCL(+)	INSR(+) PTPRB5(–) PTPN1(–)		ACTN4(+) CTNNA1(+) GNA12(+) HCLS1(+) PRKCB1(+) PRKCD(+) PRKCG(+) RAB13(+) RRAS(+) SPTAN1(+)		

Table 2 The main KEGG metabolic pathways, regulatory pathways or molecular complexes that were found to be expressed in the myometrium on the basis of genes detected in genomic studies according to ¹NP–TNIL, to ²PTNIL–TNIL or to ³TNIL–TIL, during the cycle or pregnancy. All gene transcripts showed at least a 2 fold or more, up (+) or down-regulation (–) after paired comparisons of the following stages: non-pregnant (NP) versus term not in labor (TNIL), preterm (PTNIL) versus TNIL, or TNIL versus term in labor (TIL). Brackets: number of genes represented in a defined pathway. A total of 118 genes were sorted as belonging to any of the 14 pathways, 0: no gene represented for the corresponding pathway. OMIM nomenclature adopted for genes names (see text).

Cytokine-cytokine receptors			Toll-like receptors			TFG-beta signaling pathway			Wnt signaling pathway		
NP–TNIL ¹ (8)	PTNIL–TNIL ² (12)	TNIL–TIL ³ (5)	NP–TNIL ¹ (5)	PTNIL–TNIL ² (6)	TNIL–TIL ³ (1)	NP–TNIL ¹ (6)	PTNIL–TNIL ² (2)	TNIL–TIL ³ (3)	NP–TNIL ¹ (7)	PTNIL–TNIL ² (2)	TNIL–TIL ³ (1)
CCL19(+) CCL21(+) CSF1R(+) EGFR(+) IL15RA(+) IL6(+) IL6ST(+) TNFRSF11B(+)	BLR1(+) CCL13(+) CXCR4(+) IL9(+) TNFRSF6(+) CLC(–) EGF(–) IFNGR1(–) IL6ST(–) LIFR(–) TNF(–) TNFSF4(–)	CCL13(+) CCL18(+) CXCL10(+) TGFB2(+) TNFRSF11B(+)	CD14(+) IL6(+) PIK3CA(+) RAC2(+) STAT1(+)	IKBKB(–) IRAK1(–) MAP2K4(–) MAPK10(–) PIK3CB(–) TNF(–)	CXCL10(+)	ACVRL1(+) BMP6(+) ROCK1(+) SMAD2(+) TFDP1(+) THBS2(+)	THBS2(+) TNF(–)	TGFB2(+) THBS1(+) THBS2(+)	NLK(+) PRKCB1(+) PRKCG(+) RAC2(+) ROCK1(+) SMAD2(+) TCF7L2(+)	MAPK10(–) TP53(+)	VANG1(+)

Parturition as an inflammatory process

Cytokine-chemokines interaction

There is a massive influx in the human myometrium of neutrophils, macrophages and T-lymphocytes concomitant with the onset of labor (Osman *et al.* 2003). An increase in cytokines, interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF)-alpha within tissues of the laboring uterus and cervix is demonstrated.

Chemokines enhance inflammation by inducing chemotaxis and cell activation of inflammatory cells. CXC-chemokines, attract neutrophils but not macrophages, while CC-chemokines preferentially induce migration of macrophages. Chemokine transcripts increase in myometrium at term, including CCL13 (also known as monocyte chemoattractant protein-4; MCP-4), CCL19, CCL21, CXCR4 (neuropeptide Y receptor-like; NPYRL) and CXCR5 (Burkitt lymphoma receptor) (Bethin *et al.* 2003, Charpigny *et al.* 2003, Rehman *et al.* 2003). At the time of labor, a selective increase in CXCL10 (Interferon-inducible protein-10), CCL8 and CCL13 is noted (Esplin *et al.* 2005; see Table 2). IL-8, MCP-1 and RANTES are regulated by local growth factors and cytokines such as TNF-alpha, interferon-gamma, and IL-1. IL-8 also potentiates the effect of IL-1-induced human myometrial contractions through prostaglandin E₂ (PGE₂) production at the time of parturition and up-regulates TGF-beta receptor expression in the human myometrium, suggesting an additional autocrine-signaling pathway. Therefore, it is clear that coordination of chemokine-chemokine receptor interactions plays an important role in successful pregnancy (Kayisli *et al.* 2002).

The TNF receptor super family member Fas and its cognate ligand (FasL) play a role in cell leiomyoma apoptosis (Wang *et al.* 2002). Therefore increased expression of TNF

receptor S6 (TNFRSF6; i.e. Fas receptor) in term myometrium (Table 2) await consideration in the control of the uterine growth process. Elevated expression of TNFRSF11B is observed in human myometrium with or without labor (Esplin *et al.* 2005, Rehman *et al.* 2003). A peak in uterine osteoprotegerin (i.e. OPG or TNFRSF11B), a soluble membrane bound member of the TNF-alpha receptor family that acts as a negative regulator for receptor activator of nuclear factor- κ B (RANK), has been reported during labor in the rat (Girotti & Zingg 2003). It is of note that proinflammatory agents, like TNF-alpha, have been shown to repress G-alpha-s expression in human primary myometrial cells. This repression is mediated by the RelA nuclear factor κ B (NF- κ B) subunit. However, RelA does not bind directly to the G-alpha-s promoter, suggesting repression is through a non-DNA-binding mechanism involving the coactivator, cAMP-response element binding protein-binding protein (CBP), implying that competition between individual promoters for this limiting cofactor may underpin the ability of RelA to down-regulate G-alpha-s immediately before parturition in humans (Chapman *et al.* 2005).

The gp130 protein is a subunit component of several cytokines receptors including those for leukemia inhibitory factor (LIF). Cytokines sharing the gp130 (or IL6ST) subunits are referred to as IL-6 type family of cytokines and signal through JAK/STAT pathway, reviewed in (Heinrich *et al.* 1998). They play a role in the regulation of gene activation, proliferation and differentiation. Down-regulation of IL6ST and LIF receptor at term (Table 3) may be an indication of the decrease of proliferation processes before the onset of labor.

Interferon-gamma (IFN-gamma) inhibits the proliferation of vascular smooth muscle cells and the synthesis of collagens by myofibroblasts. In human myometrial cells,

Table 3 The main KEGG metabolic pathways, regulatory pathways or molecular complexes that were found to be expressed in the myometrium on the basis of genes detected in genomic studies according to ¹NP–TNIL, to ²PTNIL–TNIL or to ³TNIL–TIL, during the cycle or pregnancy. All gene transcripts showed at least a 2 fold or more, up (+) or down-regulation (–) after paired comparisons of the following stages: non-pregnant (NP) versus term not in labor (TNIL), preterm (PTNIL) versus TNIL, or TNIL versus term in labor (TIL). Brackets: number of genes represented in a defined pathway. A total of 118 genes were sorted as belonging to any of the 14 pathways, 0: no gene represented for the corresponding pathway. OMIM nomenclature adopted for gene names (see text).

MAPK signaling pathway			Phosphatidylinositol signaling pathway			JAK/STAT signaling pathway		
NP–TNIL ¹ (15)	PTNIL–TNIL ² (12)	TNIL–TIL ³ (4)	NP–TNIL ¹ (5)	PTNIL–TNIL ² (8)	TNIL–TIL ³ (0)	NP–TNIL ¹ (6)	PTNIL–TNIL ² (7)	TNIL–TIL ³ (1)
CD14(+)	EGF(+)	BDNF(+)	IMPA2(+)	CALM1(–)		IL15RA(+)	IL9(+)	IL13(+)
EGFR(+)	IKBKB(+)	DUSP5(+)	PIK3CA(+)	CALM3(–)		IL6(+)	PIM1(+)	
FGFR1(+)	MAP2K4(+)	NR4A1(+)	PIP5K1C(+)	DGKZ(–)		IL6ST(+)	CLC(–)	
FLNA(+)	MAPK10(+)	TGFB2(+)	PRKCB1(+)	ITPR1(–)		PIK3CA(+)	IFNGR1(–)	
HSPA5(+)	PLA2G5(+)		PRKCG(+)	ITPR1(–)		PTPN6(+)	IL6ST(–)	
HSPA8(+)	RAP1A(+)			PIK3CB(–)		STAT1(+)	LIFR(–)	
MAP3K5(+)	RASA1(+)			PLCG1(–)			PIK3CB(–)	
NLK(+)	TNF(+)			PLCG2(–)				
PLA2G2A(+)	TP53(+)							
PRKCB1(+)	PLA2G2A(–)							
PRKCG(+)	PPP5C(–)							
RAC2(+)	TNFRSF6(–)							
RASGRP2(+)								
RRAS(+)								
YWHAZ(+)								

interferon gamma antagonizes IL-1beta-induced prostaglandin-endoperoxide synthase 2 (PGHS2, also more commonly known as COX-2 or PTGS2) expression and PGE₂ production (Hertelendy *et al.* 2002). Therefore, the decrease in interferon gamma receptor 1 (IFNGR1) transcript observed during the transition from PTNIL to TNIL states of the uterus (Table 2), could be interpreted as an *ad hoc* evolution for labor onset.

TLR signaling pathway

Toll-like receptors (TLRs) are evolutionarily conserved pathogen-associated microbial patterns and play important roles in innate immunity in mammals. Ten TLRs are found in the non-pregnant human uterus but TLR2 and TLR4 mRNA are expressed in the highest levels (Nishimura & Naito 2005). Among the TLR-related gene transcripts, supressor of cytokine signalling (SOCS), CD14 and interleukin-1 receptor-associated kinase 1 (IRAK) mRNAs (see Tables 2 and 4) are widely expressed in the myometrium. TLR4 mediates induction of pre-term labor (PTL) in mice treated with LPS (Wang & Hirsch 2003). In human monocytes, LPS-induced signaling through TLRs, lead to the recruitment of docking proteins such as IRAK and TNF-alpha receptor-associated factor (TRAF6) leading to the activation of IκB kinase (IKK) complex. These pathways in turn activate transcription factor such as NF-κB that coordinates the induction of genes encoding inflammatory mediators (reviewed in Guha & Mackman 2001). In human pregnant myometrial cells a positive immunoreactivity for TLR4 is observed and cells exposure to LPS lead to nuclear translocation of the p65 subunit of NFκB (Dallot *et al.* 2005). The increased expression of CD14 together with increased expression of the catalytic subunit of phosphatidylinositol 3-kinase (PI3K) in pregnant myometrium may serve to increase host protection against microbial invasion (Rehman *et al.* 2003). At the end of pregnancy, because some of the TLR-related genes such as IRAK, IKK, MKK have decreased expression compared to earlier stage of the gestation (Charpigny *et al.* 2003), it is

possible that adaptative responses to immune or growth processes take place.

TGF-beta signaling pathway

TGF-beta regulates its own expression, the expression of ECM, of matrix metalloproteinases (MMP), and of tissue inhibitor of matrix metalloproteinases (TIMP) as well as the growth of leiomyomas and of normal myometrial cells (Chegini *et al.* 1999). TGF-beta receptors mediate their action through multiple pathways, including SMAD proteins that convey TGF-beta receptor signals from the cell surface to the nucleus, resulting in transcriptional activation of TGF-beta responsive genes. TGF-beta also activates mitogen-activated protein kinase (MAPK), PKC, and calcium/calmodulin complex, inducing SMAD-independent transcriptional responses. One mechanism by which TGF-beta is activated involves thrombospondin (THBS1). THBS1 expression and protein in human myometrial tissues are increased during labor and after the administration of oxytocin (OT) (Morimoto *et al.* 1998). Myometrial transcripts of THBS1 and THBS2 increases during pregnancy (Charpigny *et al.* 2003, Rehman *et al.* 2003) and peak at the time of labor concomitantly with the expression of TGF-beta (Esplin *et al.* 2005). It is possible that the control of the TGF-beta pathway in the near term myometrium facilitates the transition of the quiescent uterus towards a contractile state, as well as a role in control of uterine growth.

Wnt signalling pathway

Leiomyomas cells in culture have high levels of both transcripts of WNT5B and of secreted frizzled related protein 1 (SFRP1), a modulator of Wnt signaling (Mangioni *et al.* 2005). Strong SFRP1 expression under high estrogenic conditions seems to contribute to the development of human uterine leiomyomas through the antiapoptotic effect of SFRP1 (Fukuhara *et al.* 2002). The increased expression of WNT5B in the pregnant

Table 4 The main KEGG metabolic pathways, regulatory pathways or molecular complexes that were found to be expressed in the myometrium on the basis of genes detected in genomic studies according to ¹NP–TNIL, to ²PTNIL–TNIL or to ³TNIL–TIL, during the cycle or pregnancy. All gene transcripts showed at least a 2 fold or more, up (+) or down-regulation (–) after paired comparisons of the following stages: non-pregnant (NP) versus term not in labor (TNIL), preterm (PTNIL) versus TNIL, or TNIL versus term in labor (TIL). Brackets: number of genes represented in a defined pathway. A total of 118 genes were sorted as belonging to any of the 14 pathways, 0: no gene represented for the corresponding pathway. OMIM nomenclature adopted for gene names (see text).

Apoptosis			Calcium signaling pathway			Neuroactive ligand-receptors		
NP–TNIL ¹ (0)	PTNIL–TNIL ² (6)	TNIL–TIL ³ (0)	NP–TNIL ¹ (8)	PTNIL–TNIL ² (8)	TNIL–TIL ³ (0)	NP–TNIL ¹ (6)	PTNIL–TNIL ² (6)	TNIL–TIL ³ (0)
	TNFRSF6(+)		EGFR(+)	BST1(+)		ADORA3(+)	GLRB(+)	
	TP53(+)		GNAQ(+)	CALM1(–)		ADRA2C(+)	GABRB1(–)	
	DFFA(–)		LYN(+)	CALM3(–)		C3AR1(+)	GABRG2(–)	
	IRAK1(–)		NFKBIB(+)	ITPR1(–)		EDN1(+)	PRGER3(–)	
	PIK3CB(–)		PHKA1(+)	PLCG1(–)		GRM2(+)	TACR2(–)	
	TNF(–)		PRKCB1(+)	PLCG2(–)		NPY(+)	TRHR(–)	
			PRKCG(+)	PTGER3(–)				
			PYGL(+)	TACR2(–)

Figure 1 Main KEGG pathways represented in the myometrium on the basis of the genes detected in genomic studies. Gene transcripts pertaining to these pathways were either up-regulated (red) or down-regulated (green) by a two fold or more factor. White boxes denote insufficiently documented links in context. See Tables 1–4 for details. (A) Comparison between the non-pregnant (NP) and term not in labor (TNIL) uterus. (B) Comparison between the preterm not in labor (PTNIL) uterus and term in labor uterus (TNIL) or between TNIL and term in labor uterus (TIL). Five pathways (Focal adhesion, MAPK, TGF-beta, JAK/STAT and apoptosis) are shown with their known links to membrane receptors (Wnt, TGF-beta, cytokines and Toll-like receptors). Three additional pathways (actin cytoskeleton, phosphatidylinositol and calcium signaling) are over-expressed in gestation by comparison to the non-pregnant state. THBS1; thrombospondin-1 (inhibitory).

myometrium suggests that the Wnt pathway is important in myometrial adaptation to pregnancy by decreasing apoptotic myometrial cell death (Rehman *et al.* 2003). Inversely, other genes associated with the inhibition of cell proliferation such as p53 are increased at the end of gestation (Charpigny *et al.* 2003).

Kinases located at the crossroad of uterine contractility and myometrial cell proliferation

MAPK-signaling pathway

MAPK signaling cascades regulate diverse processes ranging from contraction, proliferation, differentiation, and development. Five families of MAPKs have been defined in mammalian cells: i) extracellular signal-regulated kinases (ERK1 and ERK2), ii) Jun N-terminal kinases (JNK), iii) p38 kinase isozymes. ERK1 and ERK2 are activated by mitogenic stimuli such as growth factors, cytokines and phorbol esters. Members of the JNK family play crucial roles in regulating responses to various stresses and apoptosis. Among the targets of p38 MAPKs are several transcription factors, including NF- κ B, p53 and activating transcription factor 2 (ATF2), which modulate the expression of genes encoding inflammatory cytokines, see (Qi & Elion 2005). MAPKs are involved in inhibiting gap-junction-mediated cellular communication in rat myometrium (Loch-Caruso *et al.* 2003). Mechanical stretch of the rat uterus stimulates myometrial cell hypertrophy (Douglas

et al. 1988) via a mechanism involving integrin/focal adhesion/MAPK cascades (Macphee & Lye 2000). Activation of MAPKs is necessary for optimal stretch-induced c-fos expression (Oldenhof *et al.* 2002). In addition, the spatial expression of MAPK p38 and ERK-1/2 in conjunction with ATF2 isoforms within the human uterine corpus during pregnancy and labor is likely to be important in preparation of the uterus for labor (Otun *et al.* 2005).

In human myometrial cells, the MAPK pathways are also implicated in the induction of COX-2 expression (PGHS2/PTGS2; Bartlett *et al.* 1999, Sooranna *et al.* 2004) and in mediating the effects of OT (Zhong *et al.* 2003) and corticotropin-releasing hormone (CRH) (Grammatopoulos *et al.* 2000, Papadopoulou *et al.* 2004). Increasing maternal plasma levels of CRH during the last weeks of pregnancy and the substantial expression of CRH receptors in choriodecidua, placenta and myometrium suggests that this stress hormone plays a role in the control of human parturition (Sehringer *et al.* 2004). Moreover, MAPK cascade is involved in PGF_{2 α} (Ohmichi *et al.* 1997) and endothelin-1 (ET-1) (Kimura *et al.* 1999) induced rat puerperal contraction. The MAPK activity increases in the rat myometrium from day 15 to day 20 of gestation and declines sharply just before parturition (Robin *et al.* 2004). During the same interval, a shift in intracellular distribution of the Ras protein precedes the down-regulation of membrane-dependent mitogenic signaling and uterine hypertrophy as gestation approaches parturition (Ruzycky 1998). In a rat model of preterm labor, ERK

Figure 2 Main KEGG pathways represented in the myometrium on the basis of the genes detected in genomic studies. Gene transcripts pertaining to these pathways were either up-regulated (red) or down-regulated (green) by a two fold or more factor. White boxes denote insufficiently documented links in context. See Tables 1–4 for details. (A) Comparison between the non-pregnant (NP) and term not in labour (TNIL) uterus. (B) Comparison between the preterm not in labor (PTNIL) uterus and term in labor uterus (TNIL) or between TNIL and term in labor uterus (TIL). Ten pathways (focal adhesion, adherens junctions, tight junctions, actin cytoskeleton, phosphatidylinositol and calcium signaling, MAPK, TGF- β , JAK/STAT and apoptosis) are shown with their known links to cytokine, growth factors (GF) and G-protein coupled membrane receptors. CAMs; cell adhesion molecules. ECM; extra-cellular matrix.

phosphorylation levels increase, as does phosphorylation of caldesmon and of a 20-kDa myosin light chain subunit (MLC). When rats are chronically treated with an agent that prevents ERK activation, the onset of PTL is delayed (Li *et al.* 2004, Liu *et al.* 2004).

OT-mediated ERK1/2 activation in human myometrial cells involves a phospholipase C (PLC)-independent pathway (Zhong *et al.* 2003). Cross-talk between growth factor receptors and the estrogen receptor alpha (ESR1) has been proposed. The estrogen response involves estradiol (E2)-ESR1-mediated responses as well as responses resulting from convergence of growth factor and ESR1-initiated activities in the mouse uterus (Hewitt *et al.* 2005). Functional signaling proximal to IGF-IR is maintained in the ER alpha knock-out mouse uterus. ER alpha is necessary for IGF-I induction of uterine nuclear proliferative responses, and a cross-talk between IGF-IR and ER signaling pathways exists *in vivo* (Klotz *et al.* 2002).

Protein kinase C

PKC constitute a multigene family located at the crossroad of two essential uterine functions, namely contractility and cell proliferation. Six isoforms of PKC, the conventional PKC isoforms (alpha, beta1, beta2, and gamma), the novel PKC isoforms (delta, epsilon, theta, eta, lambda/iota) and the atypical PKC isoform (zeta) are evidenced in the human pregnant myometrium. Whereas protein kinase C

alpha (PRKCA) is required for proliferation of human myometrial cells (Eude *et al.* 2002), only activation of PRKCZ results in actin reorganization and elicits contractions of the human myometrium at the end of pregnancy (Di Liberto *et al.* 2003).

A balance between T helper (Th1) (pro-inflammatory) and Th2 (anti-inflammatory) cytokine production, has been described at the time of parturition, a link between TNF-alpha and IL1-beta in premature human childbirth has been proposed (Arntzen *et al.* 1998). Because, atypical PKCs are important components of the TNF/interleukin 1-beta (IL1B) signaling pathway that controls NF- κ B activation, the implication of the PRKCZ in the control of the onset of labor in women is not surprising. (Ozaki *et al.* 2003) also demonstrated that the levels of mRNA of PRKCB1 isoform in pregnant human myometrium were greater than those in nonpregnant myometrium, a feature confirmed in genomic studies (Table 3) (Rehman *et al.* 2003). The levels of the phosphorylated substrate for PKC, protein kinase C-potentiated inhibitor of protein phosphatase 17-kDa (CPI-17) which is considered to inhibit myosin light chain phosphatase was also greater in the pregnant myometrium. These results suggest that the PKC-mediated contractile mechanism is augmented in human myometrium after gestation, and that this augmentation may be attributable to the increased activity of the PRKCB1 isoform and CPI-17.

Phosphatidyl inositol signaling pathway

The PI3K cascade is activated through the binding of the regulatory subunit to phosphorylated tyrosine residues, leading to enhanced activity of the catalytic subunit. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate PIP₃ which interacts with protein kinase Akt or protein kinase B (PKB). Activation of Akt influences many cellular functions through PIP₃ binding and phosphorylation by phosphoinositide-dependent kinase 1 (PDK1). It includes cytoskeletal organization, cell growth, motility, proliferation and survival. PDK1 is known to phosphorylate PKC-zeta in the activation loop. PI3K is also activated by direct binding of the catalytic subunit to activated Ras and PI3K cascade activation can lead to the activation of the ERK cascade (reviewed in Stein & Waterfield 2000).

Increases in $[Ca^{2+}]_i$ are controlled by multiple signal pathways in myometrium. G protein coupled receptor (GPCR)-mediated stimulation of the Gq/11 subfamily and subsequent activation of PLC subfamily results in generation of IP₃, which triggers release of Ca^{2+} from the sarcoplasmic reticulum.

Five PLC isoforms: beta 1, beta 2, beta 3, gamma 1, and gamma 2 (PLCB1–4, PLCG1–2) are detected in human myometrium. OT activates human myometrium by interacting with at least two G proteins and possibly three PLC beta isoforms (Phaneuf *et al.* 1996). Indeed, the amount of PLCB4 is increased at midpregnancy, whereas PLCB1, PLCB2, and PLCB3 are up-regulated at term in the rat uterus (Mhaouty-Kodja *et al.* 2004). PLCB3 may be targeted by both contractant and relaxant signaling pathways in the human myometrium and play a critical role in the balance between them (Zhong *et al.* 2005). Both DAG-sensitive PKC, activated by PLCB products, and diacylglycerol (DAG)-insensitive PKC, possibly activated by PI3K-dependent process are involved in ERK activation to modulate rat uterine functions (Robin *et al.* 2002). In addition, protein tyrosine kinase/phosphatase activities may control both phosphorylation and activation of PLCG1 and contribute to the modulation of the generation of inositol phosphates and uterine tension.

Genomic studies exploring the transition from the NP to the TNIL state found an up-regulation of the transcripts pertaining to the phosphoinositol signaling pathway (Rehman *et al.* 2003), whereas those comparing the PTNIL to the TNIL state found a down-regulation of transcript expression (Bethin *et al.* 2003, Charpigny *et al.* 2003). This may be interpreted as a slowdown of myometrial cell proliferation processes when pregnancy comes close to term (Table 3).

Apoptosis and parturition

Proliferation of smooth muscle cells and fibroblasts occurs in early pregnancy and decreases as the uterus nears par-

turition. In late pregnancy and during involution of the rat uterus, an increase of apoptosis is observed (Leppert 1998). More recently, (Shynlova *et al.* 2006) proposed two distinct phases of myometrial growth in the rat: a myocyte hyperplasia associated with increase in anti-apoptotic proteins in the first half of gestation and a cellular hypertrophy in the second part; the transition period is characterized by caspase cascade activation for triggering the differentiation of the uterine smooth muscle. However, the extent of apoptosis or dedifferentiation that may occur in human myometrial cells during post-partum involution is still unknown. The most investigated factors linked to apoptosis are the Fas ligand (FasL), Fas receptor (FasR), the TNF-alpha and its receptors. Some of the genes associated with the inhibition of cell proliferation and differentiation are increased in the myometrium at the end of pregnancy (Table 4). For an example, the TNFR6 or Fas antigen and the p53 tumor suppressor limit cellular proliferation by inducing cell cycle arrest and apoptosis. The presence of DFFA (DNA fragmentation factor-45), known to be essential for chromatin condensation during normal apoptosis and the inhibition of the PI3K in the term pregnant myometrium, may also contribute to an increase of apoptotic events.

G-protein signaling and parturition

The silencing of the myometrial contractile function is conditioned by a predominant functional cAMP/cGMP system, whereas contractions are under the control of agonist-induced calcium mobilization via the PLC pathway. Gestational-related modifications of GPCR in the human myometrium, as well as changes in their associated kinases (GRK), cognate G proteins, and effectors have been detected (Lopez Bernal & TambyRaja 2000, Hertelendy & Zakar 2004). Some GPCRs coupled to PLC generate the second messengers IP₃ and DAG. The ensuing rise in IP₃ releases Ca^{2+} from the sarcoplasmic reticulum, causing a sudden rise in intracellular calcium, whereas DAG activates PKC and the MAPK cascades. Uterine contractility can also be enhanced via the activation of Rho GTPases, and the subsequent action of ROCKs to potentiate the effect of myosin light chain kinase (MLCK, also known as MYLK). These pathways have been discussed above. Other GPCRs coupled to the adenylyl cyclase promotes the accumulation of cAMP which controls the relaxation of the myometrium during pregnancy via the inactivation of MLCK and the activation of ATF2, a DNA-binding protein that binds to cAMP response elements (CREs) and stimulates CRE-dependent transcription (Bailey & Europe-Finner 2005). Another powerful way for increasing cAMP concentration consists of inhibiting its hydrolysis by phosphodiesterases (PDEs). Among the five PDE families (PDE 1, 2, 3, 4 and 5) identified in the human myometrium (Leroy *et al.* 1994, Leroy *et al.* 1999), one particular isoform,

PDE4B2, which specifically hydrolyses cAMP is selectively induced at the end of pregnancy suggesting a role for this protein in the setting of the contractile state of the myometrium just before delivery (Mehats *et al.* 2000, Mehats *et al.* 2001).

Neuroactive-ligands and parturition

A small selection of peptides, neuroactive factors and ion channels such as ET-1, noradrenaline, neuropeptide Y (NPY), PGE₂ and gamma-aminobutyric acid (GABA) that mediate uterine relaxation or contraction and act through GPCRs are mentioned here (see Table 4). ET-1 was first described as a potent modulator of uterine contractions – for a review see Hertelendy & Zakar (2004) – but also has mitogenic properties in humans (Breuiller-Fouche *et al.* 1998) and rat myometrial cells (Robin *et al.* 2002). ET-1 transcript is up-regulated in the pregnant myometrium (Rehman *et al.* 2003). Among the three subtypes of alpha2-adrenoceptors found in the human myometrium at term pregnancy (Adolfsson *et al.* 1998), only the alpha2C-subtype appears to be predominant in pregnant myometrium. Stimulation of GABA (A) receptors tonically inhibits contractions of the rabbit myometrium, while stimulation of GABA (B) receptors enhances contractions. Steroids interact with GABA (A) receptors to modulate uterine contractility (Majewska & Vaupel 1991). The subunit composition of GABA (A) receptor differs in rat uteri throughout gestation and just before labor in humans, a decline in GABA receptor transcripts is observed as shown in Table 4 (Bethin *et al.* 2003, Charpigny *et al.* 2003). Thyrotropin-releasing hormone-receptor (TRH-R) is expressed in term myometrium. (Fukusumi *et al.* 1995) have previously reported high level of TRH-R mRNA in the rat uterus but whether TRH plays an important role in the female reproductive tract remains to be elucidated. Prostaglandins, in conjunction with their numerous receptors activate multiple signaling pathways and exert multifaceted actions in myometrium (Hertelendy & Zakar 2004). Thus, EP3 transcripts are down-regulated in the term pregnant myometrium (Bethin *et al.* 2003, Charpigny *et al.* 2003). However, the significance of these changes remains to be established, because of the known existence of EP3 splice variants.

Future strategies for integrative analysis of myometrial functions

A question is what could be the composition of an 'ideal' genomic array for the study of parturition? The answer is not unique. 'Generic' microarrays (i.e. having the broadest representation of transcripts) seem heuristically more potent to explore and detect new interesting metabolic pathways than arrays dedicated to a specific cell type (Tierney *et al.* 2003) because parturition involves interactions of many categories of cells, even within a rather

simple tissue like the myometrium. Only in a second step, specialized arrays can be useful to investigate the physiological regulations of a precise cell population following its individualization within a given tissue. Typically, tissue and cell tests are additional.

Gene module analysis, as exemplified in this review, searches for coordinate regulation of genes that belong to a priori defined gene modules. A statistical test performed for each module relative to all other genes on the microarray calculates whether the degree of coordinate regulation is more than one would expect by chance. Therefore, a module of genes involved in a physiological process may be significantly down-regulated whereas each gene in the module under study may be transcriptionally down regulated by say only 20%, and thus not clearly detected at the individual gene level (Tierney *et al.* 2003, Wong & Chang 2005). In addition, beside classical ideas regarding trans-regulation of gene expression, a greater number of hypotheses generated from regulatory networks analysis or cis-regulatory DNA elements analysis can be validated today in a high throughput fashion using chromatin-immunoprecipitation followed by microarray analysis (ChIP-chip). For example, a large fraction of genes transcribed in the liver and pancreas have been found to bind HNF4, providing a molecular explanation for the role of HNF4 mutations and polymorphisms in hereditary and sporadic forms of diabetes mellitus (Odom *et al.* 2004).

In the majority, transcripts are not tissue-restricted, but are present to varying degrees in a wide variety of cell types – although there are exceptions, like myosin heavy chain which is primarily found in smooth muscle cells. As such, it is not necessary to create arrays only from cDNAs obtained from dedicated libraries. Many sources of clones can be used for array analyses including microarrays purchased from companies (e.g. Incyte, Affymetrix, Clontech), which consist of cDNA clones or oligonucleotides that cover a large percentage of the transcripts (known as ESTs) present in public databases (Juhasz *et al.* 2002). Only in the context of a well defined cell population, can DNA microarray data be used in a comprehensive analysis aimed at identifying the shared and unique molecular 'modules' underlying a pathological process. Thus, in peripheral organs, laser microdissection of their constitutive compartments may reveal distinct repertoires of apoptosis-associated genes, chemokines and chemokine receptors in these compartments (Shen *et al.* 2004).

Microarray technology has made it possible to widen our comprehension of the major structural and metabolic transformations which affect the myometrium from the very beginning of pregnancy until parturition. The results have proven to be coherent with the mass of information which was accumulated previously, primarily on the basis of the study of selected critical factors. Although still limited in number, the recent studies highlight the activation of precise signaling pathways, some of which may have been under evaluated. Thus, the remodelling and maturation processes that the uterus undergoes in pregnancy

appear clearly as phenomena which last during the full course of gestation. This is attested by the nature of the main signaling pathways represented, in comparison with the non pregnant-uterus versus term uterus and the comparison of the preterm uterus versus the term uterus in labor. Comparatively, the onset of labor is perhaps the phenomenon which remains the least well described by these methods of analysis, possibly because it is a phenomenon occurring in too short window to have been grasped by the few studies carried out up to now. Whatever it may be, genomic studies constitute a necessary first step of orientation which should lead to a more elaborate hierarchical vision of the physiological mechanisms of gestation, in particular by establishing new links between the generic signaling pathways that are activated during normal or pathological gestation. Genomic studies also represent an indicative step that will need to be correlated with a systematic proteomic analysis of the myometrium (Riley *et al.* 2005a, Riley *et al.* 2005b). The latter will undoubtedly develop in the very near future.

Building comprehensive strategies of genomic and proteomic analysis to explore physiologic functions remains today a challenge to which very few research groups have devoted their energy, and so far only for biological functions unrelated to the pregnant uterus (Ho *et al.* 2003). It is our hope that the merit of the studies analyzed in this review will provide enough confidence to apply this technology to far wider sample populations and enable subcategorizations on the basis of gestational length, multiple pregnancy, ethnicity and disease. This will be hardly possible without the implementation of a genuine co-operation at a larger scale amongst many research groupings.

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