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 phenomenon's 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.

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 are, as 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), human (Bethin *et al.*, 2003, Chan *et al.*, 2002, Charpigny *et al.*, 2003, Esplin *et al.*, 2005, Havelock *et al.*, 2005, Keelan *et*

50   *al.*, 2003, Marvin *et al.*, 2002a, 2002b, Tashima *et al.*, 2002), and different tissues:

51   myometrium (Aguan *et al.*, 2000, Bethin *et al.*, 2003, Chan *et al.*, 2002, Charpigny *et al.*,

52   2003, Esplin *et al.*, 2005, Havelock *et al.*, 2005, Salomonis *et al.*, 2005), fetal membranes

53   (Chen *et al.*, 2002, Keelan *et al.*, 2003, Marvin *et al.*, 2002a, 2002b, Muhle *et al.*, 2001,

54   Tashima *et al.*, 2002), trophoblast (Chen *et al.*, 2002, Kato *et al.*, 2002). Various

55   methodologies have been used: Suppression Subtractive Hybridization (SSH) (Chan *et al.*,

56   2002) or microarrays for most of the previous cited studies. All investigators have reported

57   genes differentially regulated during parturition (Romero *et al.*, 2002) and multiple novel

58   candidate markers for preterm labor (Chan *et al.*, 2002, Marvin *et al.*, 2002a, 2002b, Wu *et*

59   *al.*, 1999) or for premature rupture of membranes (Tashima *et al.*, 2002). They contributed to

60   shed light on specific pathophysiological issues like the patterns of expression of cytokines in

61   the fetal membranes and decidua particularly in the presence of intrauterine infection (Keelan

62   *et al.*, 2003) or on the induction of enzymes for prostaglandin synthesis (Bethin *et al.*, 2003).

63   Groups of genes with yet unknown functional connection to these pathologies were also

64   found coordinately expressed. This was emphasized in animal studies which allow to explore

65   the global pattern of gene expression and their co-regulation on the basis of their genomic

66   location over the full time-course of myometrial transformation (i.e. pregnancy) or following

67   experimentally-controlled infection (Girotti & Zingg, 2003, Muhle *et al.*, 2001, Salomonis *et*

68   *al.*, 2005, Wu *et al.*, 1999).

69

70   Computational methods have significantly helped to the interpretation of gene profiling

71   experiments by delineating clusters of genes sharing coherent expression features (Claverie,

72   1999). During the last five years, statistical methods and data analysis for array studies have

73   progressed enormously and today an "ideal" study should now incorporate i) careful research

74   designs (differential expression or cross sectional studies), ii) statistical methods

75 incorporating background adjustment and normalization of data, assessment of differential

76 expression after determining sample size to control the proportion of positive calls that are

77 false positives (False Discovery rate), iii) hierarchical clustering (e.g. HOPACH method)

78 (Ganesh *et al.*, 2004, Salomonis *et al.*, 2005), iv) functional organization of genes into

79 pathways networks with the aid of integrated databases like the Gene Ontology consortium

80 (Lewis, 2005) or the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, 1997,

81 Kanehisa & Goto, 2000) and finally v) integrating genotype, transcription and clinical trait

82 data, e.g. see (Salomonis *et al.*, 2005).

83

84 The biases adopted in this review are several. We focused attention on genes expressed

85 essentially in the "normal" human myometrium. One study has reported a direct comparison

86 of the non-pregnant myometrium (NP) with the pregnant human myometrium at term not in

87 labor (TNIL) (Rehman *et al.*, 2003). It gives an overall picture of the changes that the uterine

88 muscle undergoes in its adaptation to the gestation. Two studies have described the changes in

89 gene expression in the myometrium in preterm patients not in labor (PT) versus patients at

90 term not in labor (TNIL) (Bethin *et al.*, 2003, Charpigny *et al.*, 2003) and two additional

91 studies have described the changes in gene expression in the myometrium in patients at term

92 not in labor (TNIL) versus patients at term in labor (TIL) (Esplin *et al.*, 2005, Havelock *et al.*,

93 2005). Other studies have not been compiled directly, either because they did not provide

94 sufficient precise references on the genes being studied (i.e. lack of GenBank or Unigene

95 references), they considered other components of the utero-fetal unit (i.e. membranes,

96 decidua, cervix, etc.) or they were carried out on animal species. To translate lists of tens or

97 hundreds of genes found to be differentially regulated in the conditions under study into a

98 clearer understanding of the biological phenomena involved, we used both combinations of

99 searches through the literature referenced in public databases and the Onto-Tools software

100   developed by the Draghici's group at Wayne State University, Detroit (MI),

101   (http://vortex.cs.wayne.edu/Projects.html). The Onto-Express module helps recognizing

102   functional profiles (using Gene Ontology terms) for the categories: biochemical function,

103   biological process, cellular role, cellular component, molecular function, and chromosome

104   location (Draghici *et al.*, 2003). The Pathway-Express module helps data mining, by

105   proposing on the basis of a computational method a hierarchical list of several KEGG

106   pathways (Kanehisa, 1997, Kanehisa & Goto, 2000) most likely adjusted to changes observed

107   in microarray experiments (Khatri *et al.*, 2005). Drawbacks and limitations inherent to the use

108   of these tools or of their cognate alternatives for ontological analysis, have been reviewed

109   recently (Khatri & Draghici, 2005).

110

111   The variations of myometrium gene expression in the course of gestation could be dispatched

112   in 14 main KEGG pathways (http://www.genome.jp/kegg/pathway.html) that were the most

113   representatives for the changes seen in non-pregnant (NP) versus term quiescent pregnant

114   uterus (TNIL), quiescent preterm (PT) versus term quiescent uterus (TNIL) and term

115   quiescent uterus (TNIL) versus contracting uterus at labor (TIL). Tables 1-4 and Figures 1-2

116   summarize the compiled data which are commented below in more details. Wherever

117   possible, OMIM nomenclature has been adopted in the text and tables

118   (http://www.ncbi.nlm.nih.gov/omim/).

119

120   **Changes in structural and contractile genes during gestation**

121

122   Actin cytoskeleton, focal adhesion, adherens and tight junctions related genes represent a

123   large subset of genes that are over-expressed in the pregnant human myometrium as

124   compared to the non-pregnant state (Table 1).

125

126   ***Regulation of actin cytoskeleton***

127

128   Growth and cytoskeletal remodeling of myometrial cells during pregnancy are critical for

129   myometrial functions including those expressed during labor and delivery. At term, signals

130   that initiate labor, ultimately promote a switch in the phenotype in the quiescent uterus to a

131   smooth muscle which becomes spontaneously active, excitable, highly responsive to uterine

132   agonists, and exhibits a high degree of cell-cell coupling. Myometrial cells are rich in actin

133   microfilaments, intermediate filaments and microtubules that allow cells to adapt to a variety

134   of shapes and to carry out coordinated and directed movements. In rat, myometrial expression

135   of alpha-actin is high throughout pregnancy. An increased expression of gamma-actin and its

136   translocation to the membrane is observed in uterine myocytes at late gestation. Therefore, the

137   alteration in myometrial composition of contractile proteins is important to prepare the

138   myometrium for the development of contractions during labor (Shynlova *et al.*, 2005).

139

140   RhoA, a member of the Ras superfamily, and its downstream mediator RhoA kinase

141   (ROCK1) are necessary for agonist-induced stress fiber formation (Gogarten *et al.*, 2001).

142   The higher expression of protein kinase C (PKC) isoforms observed in pregnant *versus* non-

143   pregnant myometrium (Rehman *et al.*, 2003) may promote or drive the formation of stress

144   fibers. Additionally, up-regulation of ROCK1 has been demonstrated in human term

145   myometrium (Moore *et al.*, 2000, Rehman *et al.*, 2003). This leads to the idea that increased

146   endogenous ROCK1 activity, resulting in enhanced RhoA-mediated calcium sensitization, is

147   involved in the increased contractility at the time of labor. It is of note that EGF increases the

148   presence of actin and myometrial EGF receptors transcripts are increased at the time of

149   parturition (Charpigny *et al.*, 2003, Gargiulo *et al.*, 1997, Rehman *et al.*, 2003).

150

151 *Focal adhesion*

152

153 The transmission of force between the contractile apparatus of the cell and extracellular

154 matrix (ECM) occurs at membrane-associated dense plaques or "focal adhesions". Focal

155 adhesions consist of clusters of integrins that mediate interactions between the extra- and

156 intracellular environments. The cytoplasmic regions of integrins connect with cytoskeletal

157 elements and signaling components such as Focal Adhesion Kinase (FAK), while the

158 extracellular regions connect to specific extracellular matrix molecules such as collagen,

159 laminin or fibronectin.

160

161 Fetal growth imposes mechanical tension on the myometrium at term, which in turn induces

162 activation of FAK leading to focal adhesion turnover and supporting myometrial cell

163 hypertrophy. During late pregnancy, a fall in tyrosine phosphorylation of FAK and

164 stabilization of focal adhesions occur for provoking the cessation of myometrial hypertrophy.

165 Because actin polymerization and the dynamic remodeling of the actin cytoskeleton play key

166 roles in the regulation of myometrial contraction, there is growing evidence that stretching

167 induce labor, probably through a change in the expression of contraction-associated-proteins

168 (CAPs) (Macphee & Lye, 2000). In human term pregnant myometrium, changes in

169 cytoskeletal organization support a role for FAK and other focal adhesion-associated proteins

170 as regulators of actin dynamics. ITGA5 with its known partner ITGB1, is up-regulated in rat

171 myometrium during late pregnancy and labor (Williams *et al.*, 2005) as well as ITGAV in the

172 TNIL human myometrium (Rehman *et al.*, 2003). Therefore they may interact with actin-

173 binding proteins (e.g. actinin and filamin) to form mechanical links to the cytoskeleton. A

174 strong expression of vinculin is concentrated at actin-vinculin focal adhesion sites in human

175 myometrial cells (Yu & Lopez Bernal, 1998). At the time of parturition, expression of

176 integrins declines and the focal adhesion and related pathways do not change in studies which

177 compare the TNIL and TIL states of pregnancy (see Table 1).

178

179 **Parturition as an inflammatory process**

180

181 *Cytokine-chemokines interaction*

182

183 There is a massive influx in the myometrium of neutrophils, macrophages and T-lymphocytes

184 concomitant with the onset of labor (Osman *et al.*, 2003). An increase in cytokines,

185 interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF)-alpha within tissues of the

186 laboring uterus and cervix is demonstrated.

187

188 Chemokines enhance inflammation by inducing chemotaxis and cell activation of

189 inflammatory cells. CXC-Chemokines, attract neutrophils but not macrophages, while CC-

190 Chemokines preferentially induce migration of macrophages. Chemokine transcripts increase

191 in myometrium at term, including CCL13 (MCP-4 or Monocyte chemotactic protein-4),

192 CCL19, CCL21, CXCR4 (NPYRL or neuropeptide Y receptor-like) and CXCR5 (Burkitt

193 lymphoma receptor) (Bethin *et al.*, 2003, Charpigny *et al.*, 2003, Rehman *et al.*, 2003). At the

194 time of labor, a selective increase in CXCL10 (Interferon-inducible protein-10), CCL8 and

195 CC13 is noted (Esplin *et al.*, 2005) (see Table 2). IL-8, MCP-1 and RANTES are regulated by

196 local growth factors and cytokines such as TNF-alpha, interferon-gamma, and IL-1. IL-8 also

197 potentiates the effect of IL-1-induced myometrial contractions through PGE2 production at

198 the time of parturition and up-regulates myometrial TGF-beta receptors expression in vitro,

199 suggesting an additional autocrine-signaling pathway. Therefore, it is clear that coordination

200   of chemokine-chemokine receptor interactions plays an important role in successful

201   pregnancy (Kayisli *et al.*, 2002).

202

203   The TNF receptor super family member Fas and its cognate ligand (FasL) play a role in cell

204   leiomyoma apoptosis (Wang Y. *et al.*, 2002). Therefore increased expression of TNFRS6 (i.e.

205   Fas receptor) in term myometrium (Table 2) await consideration in the control of the uterine

206   growth process. Elevated expression of TNFRS11B is observed in human myometrium with

207   or without labor (Esplin *et al.*, 2005, Rehman *et al.*, 2003). A peak in uterine osteoprotegerin

208   (i.e OPG or TNFRS11B), a soluble membrane of the TNF-alpha receptor family that acts as a

209   negative regulator for receptor activator of nuclear factor-kB (RANK), has been reported

210   during labor in the rat (Girotti & Zingg, 2003).

211

212   The gp130 protein is a subunit component of several cytokines receptors including those for

213   LIF. Cytokines sharing the gp130 (or IL6ST) subunits are referred to as IL6 type family of

214   cytokines and signal through JAK/STAT pathway (Heinrich *et al.*, 1998). They play a role in

215   the regulation of gene activation, proliferation and differentiation. Down-regulation of IL6ST

216   and LIF receptor at term (Table 3) may be an indication of the decrease of proliferation

217   processes before the onset of labor.

218

219   IFN-gamma inhibits the proliferation of vascular smooth muscle cells and the synthesis of

220   collagens by myofibroblasts. In human myometrial cells, interferon gamma antagonizes IL-

221   1beta-induced prostaglandin-endoperoxide synthase 2 (COX-2 or PTGS2) expression and

222   PGE2 production (Hertelendy *et al.*, 2002). Therefore, the decrease in IFNGR1 transcript

223   observed during the transition from PT to TNIL states of the uterus (Table 2), could be

224   interpreted as an *ad hoc* evolution for labor onset.

225

226   ***TLR signaling pathway***

227

228   Toll-like receptors (TLRs) are evolutionarily conserved pathogen-associated microbial

229   patterns and play important roles in innate immunity in mammals. Ten TLRs are evidenced in

230   the uterus but TLR2 and TLR4 mRNA are expressed at the highest levels. Among the TLR-

231   related gene transcripts, SOCS, CD14 and IRAK mRNAs (see Tables 2 and 4) are widely

232   expressed in the myometrium (Nishimura & Naito, 2005). TLR4 mediates induction of PTL

233   in mice treated with lipopolysaccharide (LPS) (Wang H. & Hirsch, 2003). In monocytes,

234   LPS-induced signaling through TLRs, lead to the recruitment of docking proteins such as IL-1

235   receptor-associated kinase (IRAK) and TNF-alpha receptor-associated factor (TRAF6)

236   leading to the activation of IkappaB kinase (IKK) complex. These pathways in turn activate

237   transcription factor such as NF-kB that coordinates the induction of genes encoding

238   inflammatory mediators (Guha & Mackman, 2001). In human pregnant myometrial cells a

239   positive immunoreactivity for TLR4 is observed and cells exposure to LPS lead to nuclear

240   translocation of the p65 subunit of NFkB (Dallot *et al.*, 2005). The increased expression of

241   CD14 together with increased expression of the catalytic subunit of PI3K in pregnant

242   myometrium may serve to increase host protection against microbial invasion (Rehman *et al.*,

243   2003). At the end of pregnancy, because some of the TLR-related genes such as IRAK, IKK,

244   MKK have decreased expression compared to earlier stage of the gestation (Charpigny *et al.*,

245   2003), it is possible that adaptative responses to immune or growth processes take place.

246

247   ***TGF-beta signaling pathway***

248

249   TGF-beta regulates its own expression, the expression of ECM, of matrix metalloproteinases

250   (MMP), and of tissue inhibitor of matrix metalloproteinases (TIMP) as well as the growth of

251   leiomyomas and of normal myometrial cells (Chegini *et al.*, 1999). TGF-beta receptors

252   mediate their action through multiple pathways, including smad proteins that convey TGF-

253   beta receptor signals from the cell surface to the nucleus, resulting in transcriptional activation

254   of TGF-beta-responsive genes. TGF-beta also activates mitogen-activated protein kinase

255   (MAPK), PKC, and calcium/calmodulin complex, inducing Smad-independent transcriptional

256   responses. One mechanism by which TGF-beta is activated involves thrombospondin

257   (THBS1). THBS1 promotes replication of smooth cells and modifies responses to contractile

258   agents. THBS1 expression and protein in human myometrial tissues are increased during

259   labor and after the administration of OT (Morimoto *et al.*, 1998). Myometrial transcripts of

260   THBS1 and THBS2 increases during pregnancy (Charpigny *et al.*, 2003, Rehman *et al.*, 2003)

261   and peak at the time of labor concomitantly with the expression of TGF-beta (Esplin *et al.*,

262   2005). It is possible that the control of the TGF-beta pathway in the near term myometrium

263   facilitates the transition of the quiescent uterus towards a contractile state, as well as it

264   contributes to control the uterine growth.

265

266   ***Wnt signalling pathway***

267

268   Leiomyomas have high levels of both transcripts of WNT5B and of secreted frizzled related

269   protein 1 (SFRP1), a modulator of Wnt signaling (Mangioni *et al.*, 2005). Strong SFRP1

270   expression under high estrogenic conditions seems to contribute to the development of uterine

271   leiomyomas through the antiapoptotic effect of SFRP1 (Fukuhara *et al.*, 2002). The increased

272   expression of WNT5B in the pregnant myometrium suggests that the Wnt pathway is

273   important in myometrial adaptation to pregnancy by decreasing apoptotic myometrial cell

274   death (Rehman *et al.*, 2003). Inversely, other genes associated with the inhibition of cell

275   proliferation such as p53 are increased at the end of gestation (Charpigny *et al.*, 2003).

276

277   **Kinases located at the crossroad of uterine contractility and myometrial cell**

278   **proliferation**

279

280   *MAPK-signaling pathway*

281

282   MAPK signaling cascades regulate diverse processes ranging from contraction, proliferation,

283   differentiation, and development. Five families of MAPKs have been defined in mammalian

284   cells: i) extracellular signal-regulated kinases (ERK1 and ERK2), ii) Jun N-terminal kinases

285   (JNK), iii) p38 kinase isozymes. ERK1 and ERK2 are activated by mitogenic stimuli such as

286   growth factors, cytokines and phorbol esters. Members of the JNK family play crucial roles in

287   regulating responses to various stresses and apoptosis. Among the targets of p38 MAPKs are

288   several transcription factors, including NF-κB, p53 and ATF2, which modulate the expression

289   of genes encoding inflammatory cytokines (Qi & Elion, 2005). MAPKs are involved in

290   inhibiting gap-junction-mediated cellular communication in rat myometrium (Loch-Caruso *et*

291   *al.*, 2003). Mechanical stretch of the rat uterus stimulates myometrial cell hypertrophy

292   (Douglas *et al.*, 1988) via a mechanism involving integrin/focal adhesion/MAPK cascades

293   (Macphee & Lye, 2000). Activation of MAPKs is necessary for optimal stretch-induced c-fos

294   expression (Oldenhof *et al.*, 2002). In addition, the spatial expression of MAPK p38 and

295   ERK-1/2 in conjunction with ATF2 isoforms within the uterine corpus during pregnancy and

296   labor is likely to be important in preparation of the uterus for labor (Otun *et al.*, 2005).

297

298   In the myometrium, the MAPK pathways are also implicated in the induction of PTGS2

299   expression (Bartlett *et al.*, 1999, Sooranna *et al.*, 2004) and in mediating the effects of

300   prostaglandins (PGs) (Ohmichi *et al.*, 1997), OT (Zhong *et al.*, 2003), endothelin-1 (ET-1)

301   (Kimura *et al.*, 1999) and corticotropin-releasing hormone (CRH) (Grammatopoulos *et al.*,

302   2000, Papadopoulou *et al.*, 2004). Increasing maternal plasma levels of CRH during the last

303   weeks of pregnancy and the substantial expression of CRH receptors in choriodecidua,

304   placenta and myometrium suggests that this stress hormone plays a role in the control of

305   human parturition (Sehringer *et al.*, 2004).

306

307   MAPK activity increases in the rat myometrium from day 15 to day 20 of gestation and

308   declines sharply just before parturition (Robin *et al.*, 2004). During the same interval, a shift

309   in intracellular distribution of the Ras protein precedes the down-regulation of membrane-

310   dependent mitogenic signaling and uterine hypertrophy as pregnancy approaches parturition

311   (Ruzycky, 1998). During RU-486-induced PTL, as previously described for spontaneous

312   labor, ERK phosphorylation levels increase, as does phosphorylation of caldesmon and of 20-

313   kDa MLC. When rats are chronically treated with an agent that prevents ERK activation, the

314   onset of PTL is delayed (Li *et al.*, 2004, Liu *et al.*, 2004).

315

316   OT-mediated ERK1/2 activation in myometrial cells involves a phospholipase C (PLC)-

317   independent pathway (Zhong *et al.*, 2003). A cross-talk between growth factors receptors and

318   the estrogen receptor alpha (ESR1) has been proposed. The estrogen response involves

319   estradiol (E2)-ESR1-mediated responses as well as responses resulting from convergence of

320   growth factor and ESR1-initiated activities (Hewitt *et al.*, 2005). Functional signaling

321   proximal to IGF-1R is maintained in the ER alpha-KO mouse uterus. ER alpha is necessary

322   for IGF-1 induction of uterine nuclear proliferative responses, and a cross-talk between IGF-

323   1R and ER signaling pathways exists *in vivo* (Klotz *et al.*, 2002).

324

325   ***Protein kinase C***

326

327   PKC constitute a multigene family located at the crossroad of two essential uterine functions,

328   namely contractility and cell proliferation. Six isoforms of PKC, the conventional PKC

329   isoforms (alpha, betaI, betaII, and gamma), the novel PKC isoforms (delta, epsilon, theta, eta,

330   lambda/iota) and the atypical PKC isoform (zeta) are evidenced in the human pregnant

331   myometrium. Whereas PRKCA is required for proliferation of human myometrial cells (Eude

332   *et al.*, 2002), only activation of PRKCZ results in actin reorganization and elicits

333   contractions of the human myometrium at the end of pregnancy (Di Liberto *et al.*, 2003).

334

335   A balance between T helper (Th1) (pro-inflammatory) / Th2 (anti-inflammatory) cytokine

336   production, has been described at the time of parturition, and a link between TNF-

337   alpha/IL1-beta and premature human childbirth has been proposed (Arntzen *et al.*, 1998).

338   Because, atypical PKC's are important components of the TNF/IL1B signaling pathway that

339   controls NF-κB activation, the implication of the PRKCZ in the control of the onset of

340   labor in women is not surprising. (Ozaki *et al.*, 2003) also demonstrated that the levels of

341   mRNA of PRKCB1 isoform in pregnant human myometrium were greater than those in

342   nonpregnant myometrium, a feature confirmed in genomic studies (Table 3) (Rehman *et al.*,

343   2003). The levels of the phosphorylated substrate for PKC, CPI-17 which is considered to

344   inhibit myosin light chain phosphatase were also greater in the pregnant myometrium. These

345   results suggest that the PKC-mediated contractile mechanism is augmented in human

346   myometrium after gestation, and that this augmentation may be attributable to the increased

347   activity of the PRKCB1 isoform and CPI-17.

348

349   ***Phosphatidyl inositol signaling pathway***

350

351   The PI3-kinase cascade is activated through the binding of the regulatory subunit to

352   phosphorylated tyrosine residues, leading to enhanced activity of the catalytic subunit. PI3

353   kinase phosphorylates phosphatidyliositol-4,5-bisphosphate (PIP2) to generate PIP3 which

354   interacts with protein kinase Akt or protein kinase B (PKB). Activation of Akt influences

355   many cellular functions through PIP3 binding and phosphorylation by phosphoinositide-

356   dependent kinase 1(PDK1). It includes cytoskeletal organization, cell growth, motility,

357   proliferation and survival. PDK1 is known to phosphorylate PKC-zeta in the activation loop.

358   PI3 kinase is also activated by direct binding of the catalytic subunit to activated Ras and PI3

359   kinase cascade activation can lead to the activation of the ERK cascade (Stein & Waterfield,

360   2000).

361

362   Increases in $[Ca^{2+}]_i$ are controlled by multiple signal pathways in myometrium. GPCR-

363   mediated stimulation of the Gq/11 subfamily and subsequent activation of PLC subfamily

364   results in generation of IP3, which triggers release of Ca^{2+} from intracellular stores.

365   Five PLC isoforms: beta 1, beta 2, beta 3, gamma 1, and gamma 2 are detected in human

366   myometrium. OT activates human myometrium by interacting with at least two G proteins

367   and possibly three PLC beta isoforms (Phaneuf *et al.*, 1996). Indeed, the amount of PLCB4 is

368   increased at midpregnancy, whereas PLCB1, PLCB2, and PLCB3 are up-regulated at term in

369   the rat uterus (Mhaouty-Kodja *et al.*, 2004). PLCB3 may be targeted by both contractant and

370   relaxant signaling pathways in myometrium and play a critical role in the balance between

371 them (Zhong *et al.*, 2005). Both DAG-sensitive PKC, activated by PLCB products, and DAG-

372 insensitive PKC, possibly activated by PI3kinase-dependent process are involved in ERK

373 activation to modulate rat uterine functions (Robin *et al.*, 2002). In addition, protein tyrosine

374 kinase/phosphatase activities may control both phosphorylation and activation of PLCG1 and

375 contribute to the modulation of the generation of inositol phosphates and uterine tension.

376

377 Genomic studies exploring the transition from the NP to the TNIL state found an up-

378 regulation of the transcripts pertaining to the phosphoinositol signaling pathway (Rehman *et*

379 *al.*, 2003), whereas those comparing the PT to the TNIL state found a down-regulation of

380 transcript expression (Bethin *et al.*, 2003, Charpigny *et al.*, 2003). This may be interpreted as

381 a slowdown of myometrial cell proliferation processes when pregnancy comes close to term

382 (Table 3).

383

384 **Apoptosis and parturition**

385

386 Proliferation of smooth muscle cells and fibroblasts occurs in early pregnancy and decreases

387 as the uterus nears parturition. In late pregnancy and during involution of the rat uterus, an

388 increase of apoptosis is observed (Leppert, 1998). However, the extent of apoptosis or

389 dedifferentiation that may occur in human myometrial cells during post-partum involution is

390 still unknown. The most investigated factors linked to apoptosis are the Fas ligand (FasL), Fas

391 receptor (FasR), the TNFalpha and its receptors. Some of the genes associated with the

392 inhibition of cell proliferation and differentiation are increased in the myometrium at the end

393 of pregnancy (Table 4). For an example, the TNFR6 or Fas antigen and the p53 tumor

394 suppressor limit cellular proliferation by inducing cell cycle arrest and apoptosis. The

395 presence of DFFA (DNA fragmentation factor-45), known to be essential for chromatin

396   condensation during normal apoptosis and the inhibition of the PI3kinase in the term pregnant

397   myometrium, may also contribute to an increase of apoptotic events.

398

399   **G-protein signaling and parturition**

400

401   The silencing of the myometrial contractile function is conditioned by a predominant

402   functional cAMP/cGMP system, whereas contractions are under the control of agonist-

403   induced calcium mobilization via the PLC pathway. Gestational-related modifications of

404   GPCR in the myometrium, as well as changes in their associated kinases (GRK), cognate G

405   proteins, and effectors have been detected (Hertelendy & Zakar, 2004, Lopez Bernal &

406   TambyRaja, 2000). Some GPCRs coupled to PLC generates the second messengers IP3 and

407   DAG. The ensuing rise in IP3 releases Ca^{2+} from the sarcoplasmic reticulum, causing a

408   sudden rise in intracellular calcium, whereas DAG activates PKC and the MAPK cascades.

409   Uterine contractility can also be enhanced via the activation of Rho GTPases, and the

410   subsequent action of ROCK's to potentiate the effect of myosin light chain kinase (MYLK).

411   These pathways have been discussed above. Other GPCR coupled to the adenylyl cyclase

412   promotes the accumulation of cAMP which controls the relaxation of the myometrium during

413   pregnancy via the inactivation of MYLK and the activation of ATF-2 (Bailey & Europe-

414   Finner, 2005). Another powerful way for increasing cAMP concentration consists in

415   inhibiting its hydrolysis by phosphodiesterases (PDEs). Among the five PDE families (PDE 1,

416   2, 3, 4 and 5) identified in the human myometrium (Leroy *et al.*, 1994, Leroy *et al.*, 1999),

417   one particular isoform, PDE4B2, which specifically hydrolyses cAMP is selectively induced

418   at the end of pregnancy suggesting a role for this protein in the setting of the contractile state

419   of the myometrium just before delivery (Mehats *et al.*, 2001, Mehats *et al.*, 2000).

420

Neuroactive-ligands and parturition

A small selection of peptides, neuroactive factors and ion channels such as ET-1, noradrenaline, NPY, PGE2, GABA… that mediate uterine relaxation or contraction and act through GPCR are mentioned here (see Table 4). ET-1 exerts a stimulatory effect on myometrium contractility and proliferation. Its 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 labour in humans, a decline in GABA receptor transcripts is observed (Bethin *et al.*, 2003, Charpigny *et al.*, 2003). TRH-R is expressed in term myometrium. (Fukusumi et al., 1995) have previously reported high level of TRH-R mRNA in uterus but whether TRH plays an important role in the female reproductive tract remains to be elucidated. PGs, 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

446 A question is what could be the composition of an "ideal" genomic array for the study of

447 parturition? The answer is not unique. Undedicated or "generic" microarrays seem

448 heuristically more potent to explore and detect new interesting metabolic pathways than

449 specialized arrays could do (Tierney *et al.*, 2003). Inversely, a myometrium-dedicated

450 microarray is unlikely to provide essential clues on the mechanisms of parturition for the

451 following reasons.

452

453 Gene module analysis, as exemplified in this review, searches for coordinate regulation of

454 genes that belong to a priori defined gene modules. A statistical test performed for each

455 module relative to all other genes on the microarray calculates whether the degree of

456 coordinate regulation is more than one would expect by chance. Therefore, a module of genes

457 involved in a physiological process may be significantly down-regulated whereas each gene

458 in the module under study may be transcriptionally down regulated by say only 20%, and thus

459 not clearly detected at the individual gene level (Tierney *et al.*, 2003, Wong & Chang, 2005).

460 In addition, beside classical ideas regarding trans-regulation of gene expression, a greater

461 number of hypotheses generated from regulatory networks analysis or cis-regulatory DNA

462 elements analysis can be validated today in a high throughput fashion using chromatin-

463 immunoprecipitation followed by microarray analysis (ChIP-chip). For example, a large

464 fraction of genes transcribed in the liver and pancreas have been found to bind HNF4,

465 providing a molecular explanation for the role of HNF4 mutations and polymorphisms in

466 hereditary and sporadic forms of diabetes mellitus (Odom *et al.*, 2004).

467

468 In majority, transcripts are not tissue-restricted, but are present to varying degrees in a wide

469 variety of cell types although there are exceptions, like myosin heavy chain which is primarily

470 found in smooth muscle cells. As such, it is not necessary to create arrays only from cDNAs

471   obtained from dedicated libraries. Many sources of clones can be used for array analyses

472   including microarrays purchased from companies (e. g. Incyte, Affymetrix, Clontech, etc.),

473   which consist of cDNA clones or oligonucleotides that cover a large percentage of the

474   transcripts (known and ESTs) present in public databases (Juhasz *et al.*, 2002). Only in the

475   context of a well defined cell population, DNA microarray data can be used in a

476   comprehensive analysis aimed at identifying the shared and unique molecular 'modules'

477   underlying a pathological process. Thus, in peripheral organs, laser microdissection of their

478   several constitutive compartments may reveal distinct repertoires of apoptosis-associated

479   genes, chemokines and chemokine receptors in these compartments (Shen *et al.*, 2004).

480

481   The microarray technology made possible to widen our comprehension of the major structural

482   and metabolic transformations which affect the myometrium from the very beginning of

483   pregnancy until parturition. The results have proven to be coherent with the mass of

484   information which was accumulated previously, primarily on the basis of the study of selected

485   critical factors. Although still limited in number, the recent studies highlight the activation of

486   precise signaling pathways, some of which may had been under evaluated. Thus, the

487   remodelling and maturation processes that the uterus undergoes in pregnancy appear clearly

488   as phenomenon's which last during the full course of gestation. This is attested by the nature

489   of best represented signaling pathways, in comparisons of the non pregnant-uterus versus

490   term uterus and the comparisons of the preterm uterus versus the term uterus in labor.

491   Comparatively, the onset of labor is perhaps the phenomenon which remains the least well

492   described by these methods of analysis, possibly because it is a phenomenon occurring in a

493   too short window to have been grasped by the few studies carried out up to now. Whatever it

494   may be, genomic studies constitute a necessary first step of orientation which should lead to a

495   more elaborate hierarchical vision of the physiological mechanisms of gestation, in particular

496 by establishing new links between the generic signaling pathways that are activated during the

497 normal or pathological gestation. Genomic studies also represent an indicative step that will

498 need to be correlated with a systematic proteomic analysis of the myometrium. The latter will

499 undoubtedly develop in a very near future.

500

501 Building comprehensive strategies of genomic and proteomic analysis to explore physiologic

502 functions remains today a challenge to which very few research groups have devoted their

503 energy, and so far only for biological functions unrelated to the pregnant uterus. The

504 examples reported today indicate that this will be hardly accessible without the integrated

505 cooperation of several groups (Ho *et al.*, 2003).

506

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760

Table 1

	Actin cytoskeleton			Focal adhesion			Adherens junctions			Tight junctions		
	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]
	15	6	0	18	6	3	11	3	0	10	0	0
	ACTN4(+)	EGF(-)		ACTN4(+)	INSR(+)	ILK(+)	ACTN4(+)	INSR(+)		ACTN4(+)		
	CD14(+)	ITGAV(-)		ARHGAP5(+)	ITGB3(-)	THBS1(+)	CTNNA1(+)	PTPRB5(-)		CTNNA1(+)		
	EGFR(+)	ITGB3(-)		CAV2(+)	ITGB6(-)	TNC(+)	EGFR(+)	PTPN1(-)		GNAI2(+)		
	FGFR1(+)	ITGB6(-)		COL1A2(+)	MAPK10(-)		FGFR1(+)			HCLS1(+)		
	HEM1(+)	MATK(-)		EGFR(+)	PIK3CB(-)		NLK(+)			PRKCB1(+)		
	ITGAM(+)	PIK3CB(-)		FLNA(+)	RAP1A(-)		PTPN6(+)			PRKCD(+)		
	ITGAV(+)			ITGB5(+)			PTPRJ(+)			PRKCG(+)		
	ITGB5(+)			PIK3CA(+)			RAC2(+)			RAB13(+)		
	PFN1(+)			PIP5K1C(+)			SMAD2(+)			RRAS(+)		
	PIK3CA(+)			PRKCB1(+)			TCF7L2(+)			SPTAN1(+)		
	PIP5K1C(+)			PRKCG(+)			VCL(+)					
	RAC2(+)			ROCK1(+)								
	ROCK1(+)			RRAS(+)								
	RRAS(+)			TNC(+)								
	VCL(+)			VASP(+)								
				VCL(+)								
				VWF(+)								
				ZYX(+)								

Table 1 legend

The main KEGG (http://www.genome.jp/kegg/) metabolic pathways, regulatory pathways or molecular complexes that were found to be the best

represented in the myometrium on the basis of genes detected in genomic studies according to [1]NP-TNIL (Rehman *et al.*, 2003), to [2]PT-TNIL

(Bethin *et al.*, 2003, Charpigny *et al.*, 2003) or to [3]TNIL-TIL (Esplin *et al.*, 2005, Havelock *et al.*, 2005), 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 (PT) versus TNIL, or TNIL versus term in labor (TIL). Number of genes represented in pathways and

physiological stages, out of the total number of genes (n=118) sorted as belonging to these pathways, "0" means no gene represented for the

corresponding pathway. OMIM nomenclature adopted for gene names (http://www.ncbi.nlm.nih.gov/omim/).

Table 2

Cytokine-cytokine receptors			Toll-like receptors			TGF-beta signaling pathway			Wnt signaling pathway		
NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]
8	12	5	5	6	1	6	2	3	7	2	1
CCL19(+)	BLR1(+)	CCL13(+)	CD14(+)	IKBKB(-)	CXCL10(+)	ACVRL1(+)	THBS2(+)	TGFB2(+)	NLK(+)	MAPK10(-)	VANGL1(+)
CCL21(+)	CCL13(+)	CCL18(+)	IL6(+)	IRAK1(-)		BMP6(+)	TNF(-)	THBS1(+)	PRKCB1(+)	TP53(+)	
CSF1R(+)	CXCR4(+)	CXCL10(+)	PIK3CA(+)	MAP2K4(-)		ROCK1(+)		THBS2(+)	PRKCG(+)		
EGFR(+)	IL9(+)	TGFB2(+)	RAC2(+)	MAPK10(-)		SMAD2(+)			RAC2(+)		
IL15RA(+)	TNFRSF6(+)	TNFRSF11B(+)	STAT1(+)	PIK3CB(-)		TFDP1(+)			ROCK1(+)		
IL6(+)	CLC(-)			TNF(-)		THBS2(+)			SMAD2(+)		
IL6ST(+)	EGF(-)								TCF7L2(+)		
TNFRSF11B(+)	IFNGR1(-)										
	IL6ST(-)										
	LIFR(-)										
	TNF(-)										
	TNFSF4(-)										

Table 2 legend

See Table 1 legend

Table 3

MAPK signaling pathway			Phosphatidylinositol signaling pathway			JAK-STAT signaling pathway		
NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]	NP-TNIL[1]	PT-TNIL[2]	TNIL-TIL[3]
15	12	4	5	8	0	6	7	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(+)								

Table 3 legend

See Table 1 legend

Table 4

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

Table 4 legend

See Table 1 legend

1   **Figure legends**

2

3   **Figure 1**

4

5   Main KEGG pathways (http://www.genome.jp/kegg/) represented in the myometrium on the

6   basis of the genes detected in genomic studies. Gene transcripts pertaining to these pathways

7   were either up-regulated (red) or down-regulated (green) by a two fold or more factor. White

8   boxes denote insufficiently documented links in context. See Tables 1-4 for details. (A)

9   Comparison between the non-pregnant (NP) and term not in labor (TNIL) uterus (Rehman et

10   al., 2003). (B) Comparison between the preterm (PT) uterus and term in labor uterus (TNIL)

11   (Bethin et al., 2003, Charpigny et al., 2003) or between TNIL and term in labor uterus (TIL)

12   (Esplin et al., 2005, Havelock et al., 2005). Five pathways (Focal adhesion, MAPK, TGF-

13   beta, JAK-STAT and apoptosis) are shown with their known links to membrane receptors

14   (Wnt, TGF-beta, cytokines and Toll-like receptors). Three additional pathways (actin

15   cytoskeleton, phosphatidylinositol and calcium signaling are over-expressed in gestation by

16   comparison to the non-pregnant state. THBS1, thrombospondin-1 (inhibitory).

17

18   **Figure 2**

19

20   Main KEGG pathways (http://www.genome.jp/kegg/) represented in the myometrium on the

21   basis of the genes detected in genomic studies. Gene transcripts pertaining to these pathways

22   were either up-regulated (red) or down-regulated (green) by a two fold or more factor. White

23   boxes denote insufficiently documented links in context. See Tables 1-4 for details. (A)

24   Comparison between the non-pregnant (NP) and term not in labour (TNIL) uterus (Rehman

25   et al., 2003). (B) Comparison between the preterm (PT) uterus and term in labor uterus

26   (TNIL) (Bethin et al., 2003, Charpigny et al., 2003) or between TNIL and term in labor uterus

27   (TIL) (Esplin et al., 2005, Havelock et al., 2005). Ten pathways (focal adhesion, adherens

28   junctions, tight junctions, actin cytoskeleton, phosphatidylinositol and calcium signaling,

29   MAPK, TGF-beta, JAK-STAT and apoptosis) are shown with their known links to cytokine,

30   growth factors (GF) and G-protein coupled membrane receptors. CAMs, cell adhesion

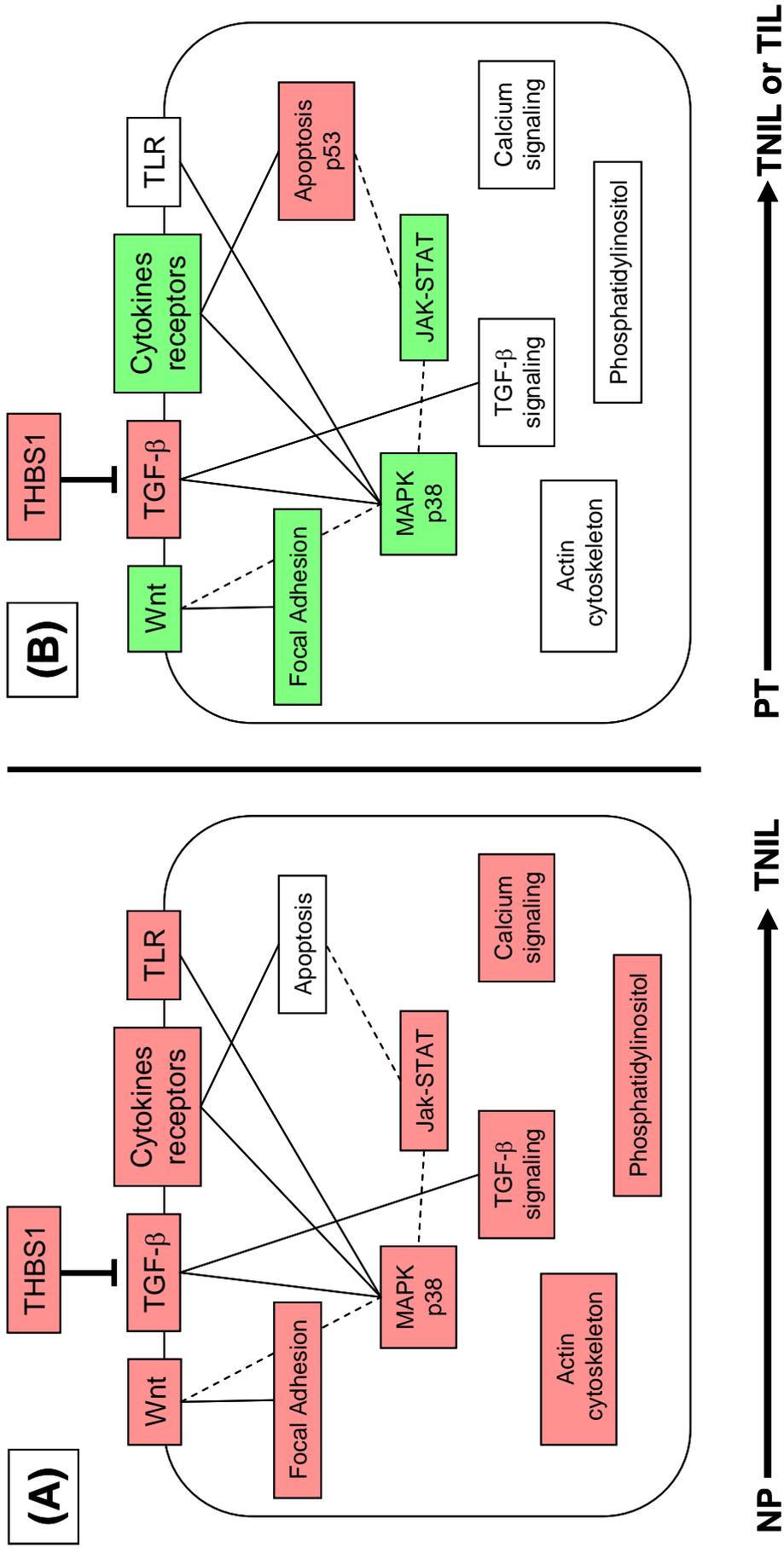31   molecules, ECM, extra-cellular matrix.

32

Figure 1

Figure 2