Class II PI3Ks α and β Are Required for Rho-Dependent Uterine Smooth Muscle Contraction and Parturition in Mice

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1 **Class II PI3K α and β are required for Rho-dependent uterine smooth muscle**

2 **contraction and parturition in mice**

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15 parturition

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Abstract

Class II phosphoinositide 3-kinases (PI3K), PI3K-C2α and PI3K-C2β, are highly

homologous and distinct from class I and III PI3K in the catalytic products and domain

structures. In contrast to class I and class III PI3Ks, physiological roles of PI3K-C2α

and PI3K-C2β are not fully understood. Because we previously demonstrated that

PI3K-C2α is involved in vascular smooth muscle contraction, we studied the

phenotypes of smooth muscle-specific knockout (KO) mice of PI3K-C2α and

PI3K-C2β. The pup numbers born from single PI3K-C2α-KO and single PI3K-C2β-KO

mothers were similar to those of control mothers, but that from double KO (DKO)

mother was smaller compared with control mice. However, the number of intrauterine

fetuses in pregnant DKO mothers was similar to that in control mice. Both spontaneous

and oxytocin-induced contraction of isolated uterine smooth muscle (USM) strips was

diminished in DKO mice but not either of the single KO mice, compared with control

mice. Furthermore, contraction of USM of DKO mice was less sensitive to a Rho kinase

inhibitor. Mechanistically, the extent of oxytocin-induced myosin light chain

phosphorylation was greatly reduced in USM from DKO mice compared with control

mice. Oxytocin-induced rise in the intracellular Ca^{2+} concentration in USM was similar

in DKO and control mice. However, Rho activation in the intracellular compartment

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55   was substantially attenuated in DKO mice compared with control mice, as evaluated by

56   fluorescence resonance energy transfer imaging technique. These data indicate that both

57   PI3K-C2α and PI3K-C2β are required for normal USM contraction and parturition

58   mainly through their involvement in Rho activation.

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Introduction

Reproduction is highly coordinated by various hormones and local mediators with the integration by the neuroendocrine system (1,2). The uterus, one of the most important female reproductive organs, accommodates and nurtures the growing fetus (3). The uterus comprises three layers: the endometrial, myometrial and outer serosal layers. During the pregnancy, the myometrium remains quiescent and undergoes enormous hyperplasia and hypertrophy to provide proper environment for fetal growth and to prepare for the generation of uterine contractile force sufficient for fetal delivery. At the last stage of pregnancy, stretching of the uterine wall by the grown-up fetus and other changes in the local uterine environment causes the activation of the myometrium, bringing about myometrial alterations including the extreme upregulation of oxytocin receptor and voltage-dependent Ca^{2+} channel (4,5).

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that catalyze the phosphorylation at 3'-hydroxyl group of the inositol ring in phosphoinositides (6). PI3Ks comprise three classes of PI3Ks; class I PI3Ks, p110α, p110β, p110γ and p110δ, are activated downstream of various receptor tyrosine kinases and G protein-coupled receptors, to mainly generate phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P$_3$) and to mediate cell proliferation, survival and migration. Class III PI3K, Vps34, mainly

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91 regulates autophagy by generating phosphatidylinositol 3-phosphate (PI(3)P). Class II

92 PI3Ks comprise PI3K-C2α (C2α), PI3K-C2β (C2β), and PI3K-C2γ (C2γ), which

93 generate phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) and probably PI(3)P (7-9).

94 C2α and C2β are ubiquitously expressed widely in various organs and tissues whereas

95 C2γ expression is restricted mainly in liver, breast, testis and prostate (10). In contrast to

96 class I and class III PI3Ks, the physiological function of class II PI3Ks was not well

97 understood.

98   We previously demonstrated using siRNA-mediated specific knockdown technique

99 that C2α was required for noradrenaline- and ionomycin-induced contraction of

100 vascular smooth muscle cells (11-13). Subsequently, we generated C2α-knockout (KO)

101 mice to study a role of C2α at an organismal level and found that homozygous C2α-KO

102 mice were embryonic lethal due to severe vascular endothelial defects (14).

103 C2α-deficient vascular endothelial cells showed impairment of Rho activation in the

104 endosomes in response to the angiogenic factor VEGF due to defects of VEGF receptor

105 internalization, which led to impaired proliferation, migration and cell-cell adhesion of

106 endothelial cells (14). Furthermore, we observed in endothelial cells that endosomal

107 activation of Rac and Smad2/3 was also impaired upon stimulation by

108 sphingosine-1-phosphate (S1P) and TGFβ1, respectively, due to defects in the

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109   internalization of S1P receptor and TGFβ1 receptor (9,15). These findings suggested

110   that C2α is required for receptor endocytosis and subsequent receptor signaling in the

111   endosomes for at least certain receptor ligands in endothelial cells. C2β is highly

112   homologous in its amino acid sequence to C2α and exhibits similar activities including

113   cell migration and growth compared with C2α. However, it is little known how

114   important C2β is in endocytosis and the intracellular signaling (16).

115   In the present study, we sought to reveal physiological roles of C2α and C2β in

116   smooth muscle organs by generating smooth muscle-specific KO mice of C2α and C2β.

117   During this study, we found that pregnant smooth muscle-specific double KO (smDKO)

118   female mice, but not single KO mice of either C2α or C2β, delivered smaller numbers

119   of pups compared with control mice although the numbers of fetuses in the uteri at the

120   term pregnancy did not differ between smDKO and control mice. Isolated uterine

121   smooth muscle from smDKO mice showed attenuated contraction with reduced

122   intracellular Rho activation, compared with control mice. These results indicate the

123   novel physiological role of C2α and C2β in uterine smooth muscle contraction and pup

124   delivery.

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Materials and Methods

Materials

Advanced DMEM (Gibco, cat no. 12491-015) and FluoroBrite DMEM (Gibco, cat no. A1896701) were purchased from Gibco/Life Technologies Corporation, NY. Fluo-8 AM (cat no. 21081) was purchased from AAT Bioquest, Sunnyvale, CA. Liberase TM (cat no. 5401127001) and protease inhibitor cocktail Complete mini (Cat. no. 11836153001) were purchased from Roche, Mannheim, Germany. Y-27632 (Cat no. 253-00513) and normal goat serum (cat no. 143-06561) were purchased from Wako Pure Chemicals Industries, Osaka, Japan. Oxytocin (cat no. O6379), nitrendipine (cat no. N144) and other chemicals were bought from Sigma-Aldrich, Saint Louis, MO unless others specified. The following antibodies were used in this study: rabbit polyclonal PI3K-C2α (cat no. AP1155B, Abgent, San Diego, CA), rabbit polyclonal PI3K-C2β (cat no. sc-134766, Santa Cruz, Dallas, TX), rabbit polyclonal anti-smooth muscle myosin heavy chain 11 (cat no. ab53219, Abcam, Cambridge, UK), mouse monoclonal anti-caldesmon (cat no. C-6542, Sigma-Aldrich, Saint Louis, MO), monoclonal anti-myosin light chain kinase (MLCK) (cat no. M 7905, Sigma-Aldrich, Saint Louis, MO), rabbit polyclonal PP1δ antibody (17), mouse monoclonal anti-calponin (cat no. C2687, Sigma-Aldrich, Saint Louis, MO), rabbit polyclonal phospho-myosin

145   phosphatase target subunit-1 （MYPT1) (Thr^{853}) (cat no.4563, Cell Signaling

146   Technologies, Danvers, MA), mouse monoclonal anti-MYPT1 (cat no. 612165, BD

147   Biosciences San Jose, CA), rabbit monoclonal PI3 Kinase Class II α (D3Q5B) from Cell

148   Signaling Technologies, Danvers, MA (Ref#12402), mouse monoclonal anti-PI3K-C2β

149   antibody from BD Biosciences, San Jose, CA (Material number 611342), rabbit

150   polyclonal phospho-MLC$_{20}$ (Ser^{19}) (cat no. 3671, Cell Signaling Technologies, Danvers,

151   MA), mouse monoclonal anti-myosin light chain (product no. M4401, Sigma-Aldrich,

152   Saint Louis, MO), mouse monoclonal anti-actin, α-smooth muscle (cat no. A5228,

153   Sigma-Aldrich, Saint Louis, MO), rabbit polyclonal di-phosphorylated MLC$_{20}$

154   (Thr^{18}/Ser^{19}) from Dr. M. Seto in Asahi chemical industry (Fuji, Japan), mouse

155   monoclonal anti-calcium channel L-type DHPR alpha subunit (Cav$_{α2}$) (cat no. ab2864,

156   Abcam, Cambridge, UK), mouse monoclonal anti-Slo1 (BKα1) (cat no. MABN70,

157   EMD Millipore, Billerica, MA).

158

159   **Mice**

160   All animal experiments were conducted according to the Fundamental Guidelines

161   for Proper Conduct of Animal Experiment and Related Activities in Academic Research

162   Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science

163   and Technology of Japan, and were approved by the Committee on Animal in

164   Kanazawa University. PI3K-C2α-floxed (C2α$^{fl/fl}$) mice were described previously (14).

165   PI3K-C2β mice that carry 3 loxP sequences, one loxP before exon 3 and two loxP

166   sequences flanking the neomycin cassette (neo) in the recombinated *Pik3c2b* gene

167   (C2β$^{3lox/3lox}$ mice) (Supplementary Fig. 1) (18) were obtained from the Jackson

168   laboratory (B6.129-*Pik3c2b^{tm1Pkha}*/J, stock No. 005702) and described elsewhere (19).

169   To generate mice with *Pi3kc2b*-conditional allele that carries two loxP sites (C2β$^{fl/fl}$

170   mice), C2β$^{3lox/3lox}$ mice were crossed with Insulin-Cre recombinase (Cre) transgenic

171   mice to delete the neo (Supplementary Fig. 1) (14,18). To generate *Pi3kc2b* null (C2β$^{-/-}$)

172   mice, 3 loxP-targeted allele was removed by intercrossing the CAG-Cre transgenic mice

173   line (20) (Supplementary Fig. 2) (18). To generate smooth muscle-specific

174   *Pik3c2b*-conditional KO (C2β$^{fl/fl}$; SM22α-Cre) mice, C2β$^{fl/fl}$ mice were crossed with

175   SM22α-Cre transgenic mice (21) (Supplementary Fig. 1) (18) . In some experiments,

176   C2β$^{3lox/3lox}$ mice were crossed with SM22α-Cre transgenic mice to generate smooth

177   muscle-specific C2β$^{-/-}$ mice (Supplementary Fig. 3) (18). In the present study, we

178   employed three genetically different mice with C2α- and C2β-double KO in smooth

179   muscle, i.e. C2α$^{fl/fl}$; C2β$^{fl/fl}$; SM22α-Cre, C2α$^{fl/fl}$; C2β$^{3lox/3lox}$; SM22α-Cre, and C2α$^{fl/fl}$;

180   C2β$^{-/-}$; SM22α-Cre mice (Supplementary Figures 1-3)(18), to study roles of C2α and

181 C2β. Rosa26-CAG-loxP-stop-loxP-tdTomato (R26-tdTomato) reporter mice were from

182 the Jackson Laboratory (B6;129S6-*Gt(ROSA)$^{26Sortm14(CAG-tdTomato)Hze}$*/J, Stock No.

183 007908). SM22α-Cre; R26-tdTomato mice were generated to evaluate Cre-mediated

184 recombination efficiency. Mice were sacrificed using intraperitoneal injection (*i.p.*) of

185 pentobarbital (Kyoritsu, Tokyo, Japan) overdose according to the acceptable *euthanasia*

186 guidelines. Mice were genotyped by PCR analysis of genomic DNA prepared from tail

187 biopsies.

188

189 **Counting pups and intrauterine fetuses**

190   We performed timed mating of mice. Mouse mating was performed in 1:1 ratio by

191 placing one female mouse into a cage containing one male mouse at late afternoon at

192 gestational day (GD) 0. The following morning, all female mice, whether they were

193 containing vaginal plug or not, were separated from male mice and followed until

194 GD18.5 or delivery. We counted delivered pups on the morning of the expected day of

195 delivery. In some pregnant female mice, we carefully monitored numbers of pups

196 delivered from each mouse every three hours on the expected delivery day. For counting

197 intrauterine fetuses in the fertility test, mice were sacrificed at GD 18.5 with overdose of

198 pentobarbital and intrauterine fetuses were counted. In the fertility test, 12 control and

199   12 double KO mice were used and the data was from 5 litters in both control and

200   smDKO female mice.

201

202   **Western blotting**

203   For preparation of non-stimulated myometrium tissue lysate, myometrium layer was

204   quickly dissected from the isolated uterus by removing the endometrium and placenta

205   after sacrificing mice. The myometrial tissues were snap-frozen by soaking them in

206   liquid nitrogen. Frozen tissues were quickly homogenized with glass homogenizer in ice

207   cold modified RIPA buffer (10 mM Tris-HCl, pH7.4, 100 mM NaCl, 1 mM EDTA,

208   1mM EGTA, 1 mM NaF, 20 mM Na_2HPO_4, 2 mM Na_3VO_4, 0.1% SDS, .05% Sodium

209   deoxycholate, 1% Triton-X 100, 10% glycerol, and one tablet of complete mini per 10

210   ml buffer) or kept at -80°C for storage. After debris was removed, the samples were

211   solubilized in 2 times concentrated Laemmli's SDS sample buffer, boiled for 5 mins,

212   and then separated on 8% or 14% SDS-PAGE, followed by electrotransfer to PVDF

213   membranes (Immobilon-P, Millipore-Merck, Darmstadt, Germany). After blocking with

214   5% bovine serum albumin (BSA) for 1 h, the membranes were incubated overnight with

215   different antibodies at 4 °C, followed by incubation with alkaline

216   phosphatase-conjugated secondary antibodies for 1 h at room temperature. Protein

217 bands were visualized by color reaction using nitro blue tetrazolium/

218 5-bromo-4-choloro-3'-indolylphosphate p-toluidine system. The band densities of

219 different proteins were determined, using Image Studio lite software (LI-COR).

220

221 **Immunofluorescent staining**

222   Cryosections of uterus were stained for immunofluorescence observations, using

223 standard protocol as described previously (14). Briefly, after overnight fixation in 4%

224 PFA in Ca^{2+} and Mg^{2+} free PBS, the tissues were washed several times with PBS,

225 cryoprotected in 20% sucrose for 12-16 h and snap frozen with TissueTek optimal

226 cutting temperature (OCT) compound (Sakura, Tokyo, Japan). Tissue sections (7 µm)

227 were prepared by using Sakura Tissue-Tek Cryo3 and the sections were blocked with

228 5% normal goat serum in 0.3% Triton-X/PBS for 1h at room temperature. Primary

229 antibodies were applied at the indicated dilutions: anti-PI3K-C2α (1:100), anti-

230 PI3K-C2β (1:200), anti-Mhc11 (1:250), anti-calcium channel L-type DHPR alpha

231 subunit (Cavα$_2$) (1:100), anti-Slo1 (BKα1) (1:150) and incubated for 12-16 h at 4°C.

232 After several washes by PBS, Alexa Fluor 488 conjugated anti-rabbit IgG (1:1000) was

233 applied to the sections and incubated at room temperature for 2h. Sections were

234 counterstained with DAPI for 30 mins and mounted with Fluoromount (Diagnostic

13

235 BioSystems, Pleasanton, CA) with coverslips. Confocal microscopic observations were

236 carried out on an inverted Nikon Eclipse Ti2 confocal microscope (Nikon Instruments),

237 attached to an Andor Dragonfly spinning-disk unit, Andor EMCCD camera (iXon

238 DU888) (Andor Technologies) and a laser unit (Coherent). An oil-immersion objective

239 (PlanApo 60X, NA 1.4) (Nikon) was used for all experiments. Excitation for DAPI,

240 Alexa488 and Alexa568 chromophores was provided by 405, 488 and 561 nm laser,

241 respectively. Super-resolution imaging of fixed cells was performed using an Andor

242 Dragonfly confocal microscope in SRRF-Stream mode.

243

244 **Measurements of tension and phosphorylation of MLC_{20} and MYPT1**

245   Tension measurements were performed as described previously (22). In brief,

246 isolated uteri were placed immediately in ice-cold Krebs-Henseleit buffer composed of

247 (in mM) NaCl 119, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.5, $CaCl_2$ 1.5, $NaHCO_3$ 25 and

248 glucose 11. The buffer containing uterine tissues was continuously aerated with 95% O_2

249 and 5% CO_2. Uteri were cleaned of fetus, placenta and other adherent tissues and made

250 strips of about 2.5mm x 15mm length. After placing strips in contraction chambers,

251 generated tension was determined isometrically with transducer (UM–203; Kishimoto

252 Medical Instruments, Kyoto, Japan) with continuously aerated by 95% O_2 and 5% CO_2.

253   Before test stimulation, the rings were precontracted with 60 mM KCl containing buffer

254   and poorly responsive strips were not used for analyses. Spontaneous tension and

255   oxytocin- and KCl-induced tension were corrected by the tension generated by the

256   application of a hypotonic buffer at the end of tension measurements as described

257   previously (23,24). The optimal concentrations of oxytocin, Y-27632 and nitrendipine

258   were determined by preliminary experiments.

259   Uterine strips contracted isometrically were fixed in acetone dry ice slurry

260   containing 20 mM dithiothreitol (DTT) and 10% trichloroacetic acid and washed in

261   acetone containing 10mM DTT at room temperature, as described previously (12).

262   Fixed tissues were homogenized in a homogenization buffer comprising 20 mM

263   Tris/HCl (pH 7.5), 100 mM NaF, 1 mM Na_3VO_4, 0.1% SDS, 2 mM EGTA, 0.5%

264   NP-40, 20 μg/ml each of leupeptin and aprotinin and 1 mM PMSF. The homogenates

265   were mixed with 4 times concentrated Laemmli's SDS sample buffer and boiled for 5

266   minutes. The samples (40 μg protein) were separated on 8% and 14% SDS-PAGE,

267   followed by western blot analysis using phospho-MLC_{20} and -MYPT1 specific

268   antibodies and antibodies that recognize both phospho- and nonphospho-proteins. The

269   amounts of the phospho-proteins quantitated by densitometry were normalized for total

270   amounts of MLC_{20} and MYPT1in each sample, and the quantified data of normalized

271 amounts of the phospho-proteins were expressed as multiples over a value in

272 unstimulated tissues, which is expressed as 1.0.

273

274 **Isolation and culture of myometrial cells**

275 Myometrial cells were isolated and cultured as described previously (25). Briefly,

276 the uterus quickly removed from euthanized mice was washed with ice-cold Hank's

277 balanced salt solution (HBSS) and cut through the longitudinal axis. The placenta, fetus

278 and endometrium were removed from the uterus and the myometrium layer was finely

279 chopped with scissors. The myometrial tissues were incubated in 1 unit/ml Liberase-TM

280 in HBSS solution for 1 h at 37 °C. Cell suspension was prepared by gently pipetting

281 15-20 times with penicillin G- and strepromycin-supplemented, 5% FBS-containing

282 advanced DMEM and filtered through 100 µm Cell Strainer (cat no. 352360, Falcon,

283 Corning, NY). Cell suspension was centrifuged at 450 x g for 5 min at 20 °C, and the

284 resultant cell pellet was resuspended in penicillin G- and strepromycin- supplemented,

285 5% FBS-containing advanced DMEM and plated onto type I collagen (Nitta

286 gelatin)-coated dishes.

287

288 **Measurements of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)**

289   For measurements of the $[Ca^{2+}]_i$, freshly isolated myometrial smooth muscle cells

290   were seeded on type I collagen-coated glass-bottomed culture dishes (cat no.

291   P35G-1.0-14-C, Mat Tek Corp.) and kept for 18-20 h at 37°C in the atmosphere

292   containing 5 % CO_2. Cells were washed with pre-warmed HBSS and loaded by 5 µM of

293   Fluo-8 AM in HBSS for 30 mins. After washing twice by HBSS, cells were incubated in

294   phenol red-free FluoroBrite DMEM (Gibco) on a heated stage chamber at 37 °C

295   temperature and 5% CO_2 atmosphere (Tokai-Hit). The intracellular Ca^{2+}-imaging was

296   performed using a customized inverted microscope (IX70, Olympus)-based spinning

297   disk (CSU-10, Yokogawa) confocal system, equipped with an EM-CCD cooled

298   charge-coupled devise camera (iXon, Andor, UK) and a light engine (Lumencor, Inc.).

299   Fluorescent images were captured every 500 ms with excitation at 488 nm light and

300   fluorescence detection at 510 nm. Pixel density was calculated from whole cell averages

301   using the iXon iQ software (Andor). The ratio of oxytocin stimulated peak fluorescence

302   intensity (F) / basal intensity (F0) was expressed.

303

304   **Determination of Rho activation by fluorescence resonance energy transfer**

305   **(FRET) imaging technique**

306   For FRET imaging analysis, after myometrial smooth muscle cells were isolated,

307   they were transfected with the pRaichu-RhoA probe (26) using an Amaxa Nucleofector

308   system (Lonza) and plated onto type I collagen-coated glass-bottomed culture dishes.

309   Twenty-four h later, the transfected cells were imaged using the same basic confocal

310   microscope system as described for the $[Ca^{2+}]_i$ measurements. For the measurements of

311   Rho-FRET signals, the confocal system was configured with a CFP and YFP filter set

312   (Di01-T445/515/561-13×15×0.5, Semrock). The employed chimeric FRET probe

313   protein consists of N-terminal yellow fluorescent protein (YFP), the Rho-binding

314   domain of PKN, RhoA, and C-terminal cyan fluorescent protein (CFP). When RhoA in

315   the chimeric FRET probe protein is bound to GDP, fluorescence of 475 nm emanates

316   from CFP with excitation at 433 nm. When RhoA is bound to GTP, intramolecular

317   binding of GTP-loaded RhoA to the RBD brings YFP into close proximity to CFP,

318   which causes FRET and fluorescence of 527 nm from YFP. The chimeric FRET

319   probe-transfected cells were stimulated with oxytocin (100 nM), which was added after

320   2 min observations of baseline signals. Pseudo-grayscale ratio images were generated

321   from images from CFP and FRET channels using Andor iQ software. RhoA FRET

322   signals intensity within 4 subcellular regions per cell at 3 min after the additions of

323   oxytocin was quantified. The ratio of oxytocin stimulated fluorescence intensity (F) /

324   basal intensity (F0) is expressed.

325

326 **Statistics**

327   Statistical analysis and graphical presentation were performed with Prism 7 software

328 (GraphPad Software). Data are presented as means ± standard error of mean (SEM).

329 Analysis between two groups was done with 2-tailed unpaired Student's t-test. For

330 comparison between multiple groups, one or two ways ANOVA followed by Bonferroni

331 post hoc test was used unless stated otherwise. p value < 0.05 was considered to be

332 statistically significant.

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343 **Results**

344 **Genetic deletion of C2α and C2β in female mice impedes fetal delivery**

345   In order to study roles of C2α and C2β in the smooth muscle organs, we generated

346 smooth muscle-specific KO mice of C2α and C2β by mating C2α- and C2β-floxed mice

347 with SM22α-Cre transgenic mice. In this study, we used two different types of

348 C2β-floxed mice, i.e. C2β$^{fl/fl}$ and C2β$^{3lox/3lox}$ mice (Supplementary Figs. 1 and 3C) (18),

349 to delete C2β gene specifically in smooth muscle tissues. We confirmed that SM22α

350 promoter-driven Cre expression effectively deleted the floxed gene in the

351 R26-tdTomato reporter construct in smooth muscle tissues including uterus and bladder

352 (Fig. 1A). SM22α promoter-driven Cre expression substantially decreased the protein

353 expression of both C2α and C2β in the myometrial layer of the uterus (Fig. 1B) of

354 smDKO mice with the genotype of C2α$^{fl/fl}$;C2β$^{fl/fl}$;SM22α-Cre. While we were

355 conducting the mating, we found that female smDKO mice in the mating

356 (C2α$^{fl/fl}$;C2β$^{fl/fl}$;SM22α-Cre (female) x C2α$^{fl/fl}$;C2β$^{fl/fl}$ (male)) delivered reduced

357 numbers of pups compared with two other mating (C2α$^{fl/fl}$;C2β$^{fl/fl}$ (female) x

358 C2α$^{fl/fl}$;C2β$^{fl/fl}$;SM22α-Cre (male), and C2α$^{fl/fl}$;C2β$^{fl/fl}$ (female) x C2α$^{fl/fl}$;C2β$^{fl/fl}$ (male))

359 (Table 1). We also observed a reduction in the number of pups from female

360 C2α$^{fl/fl}$;C2β$^{-/-}$;SM22α-Cre mice, in which C2β is globally deleted, compared with female

361   C2α$^{fl/fl}$;C2β$^{-/-}$ mice (Table 2). C2β$^{3lox/3lox}$ mice expressed approximately 80% of the C2β

362   protein expression level in smooth muscle tissues of wild-type mice. The pup number

363   from female C2α$^{fl/fl}$;C2β$^{3lox/3lox}$;SM22α-Cre mice was also reduced compared with

364   female wild-type and C2α$^{fl/fl}$;C2β$^{3lox/3lox}$ mice (Table 3). In contrast, single smooth

365   muscle-specific KO of C2α (smC2αKO) or C2β (smC2βKO) in female mice did not

366   reduce pup numbers (Table 3). We followed the time course of the delivery in pregnant

367   mice: both control (C2α$^{fl/fl}$;C2β$^{fl/fl}$) and smDKO mice started delivery at night or early

368   morning. The control mice finished the delivery process by 15:00 whereas smDKO

369   mice delivered the small number of pups by 15:00 and thereafter smDKO mice did not

370   deliver pups or delivered only a few pups by 24:00 (Fig. 1C). Regardless of the

371   decreased pups delivered from smDKO mice, the number of fetuses at gestational day

372   (GD) 18.5 in the uterus of smDKO mice was not different from that of control

373   C2α$^{fl/fl}$;C2β$^{fl/fl}$ mice (Fig. 1D). The gross fetal appearance including the attachment and

374   orientation of fetuses to the placenta in smDKO pregnant mice were also similar to that

375   in control mice. These observations suggested that the fetal delivery process was

376   impaired in female smDKO mice.

377

378   **Contraction of uterine smooth muscle from smDKO mice is impaired**

379   The gross structure of the uterus was similar in female control and smDKO mice.

380   The myometrium in the uterus of smDKO mice showed the well-developed outer

381   longitudinal and inner circular layers with normal thickness compared with control

382    $C2\alpha^{fl/fl};C2\beta^{fl/fl}$ mice (Fig. 2 A and B). The endometrium in smDKO mice also appeared

383   normal microscopically compared with control $C2\alpha^{fl/fl};C2\beta^{fl/fl}$ mice (Fig. 2B). In

384   addition, the expression levels of different contractile and smooth muscle specific

385   proteins including smooth muscle-specific myosin heavy chain Mhc11, MLCK, the

386   actin filament-associated caldesmon and calponin, the catalytic subunit of myosin light

387   chain (MLC_{20}) phosphatase $PP1\delta$ and the myosin-binding regulatory subunit of MLC_{20}

388   phosphatase MYPT1were all similar in the uteri of control and smDKO mice at both the

389   non-pregnant and pregnant (GD18.5) stages (Fig. 2C). The expression of $C2\alpha$ protein in

390   the myometrium of control mice was also similar at the non-pregnant and pregnant

391   (GD18.5) stages (Fig. 2D). $C2\beta$ protein in pregnant control mice tended to be lower

392   compared with non-pregnant control mice. The expression of $C2\alpha$ and $C2\beta$ in smDKO

393   mice was substantially reduced at the non-pregnant and pregnant stages compared with

394   control mice.

395   We next studied the possibility that smDKO pregnant mice might have impaired

396   uterine contraction, which led to the impeded delivery. We prepared uterine strips from

22

397   control and KO mice at GD18.5 and compared contractile responses. The uterine strips

398   showed spontaneous, repeated contraction of the duration of several seconds under the

399   isometric condition. The amplitude and frequency of spontaneous contraction were both

400   reduced in uterine strips from smDKO mice compared with those from control mice

401   (Fig. 3A upper and Fig. 3A lower). The uterine strips from single C2α KO (smC2αKO)

402   mice showed similar amplitudes and frequency of spontaneous contraction to those of

403   control mouse strips. The uterine strips from single C2β KO (smC2βKO) mice showed

404   similar amplitude, but lower frequency of spontaneous contraction compared with

405   control mice. Similar to the case of spontaneous contraction, the amplitudes of

406   contractile responses induced by oxytocin and KCl, which activate phospholipase

407   C/Rho pathways and L-type voltage-dependent Ca^{2+} channel, respectively, were also

408   diminished in smDKO but not smC2αKO and smC2βKO uterine strips compared with

409   control mice (Fig. 3 B and C). Oxytocin and KCl increased spike frequency to the

410   similar extents in control, smC2αKO, smC2βKO and smDKO strips (lower panels of

411   Fig. 3B and C), compared with oxytocin- and KCl-nonstimulated strips (lower panel of

412   Fig. 3A).

413   To explore the mechanisms by which uterine contraction is dependent on C2α and

414   C2β, we compared the responses of uterine contraction to the L-type voltage-dependent

23

415   Ca^{2+} channel antagonist nitrendipine and the Rho kinase inhibitor Y-27632 in control

416   and smDKO strips. Nitrendipine substantially inhibited spontaneous contraction in both

417   control and smDKO strips. Nitrendipine relatively weakly inhibited oxytocin-induced

418   contraction in control and smDKO strips compared with spontaneous contraction. These

419   observations suggest that both L-type Ca^{2+} channel-dependent and -independent

420   mechanisms are involved in spontaneous and oxytocin-induced contraction in control

421   and smDKO uterine smooth muscle. The amplitudes of spontaneous and

422   oxytocin-induced contraction in the presence of nitrendipine were similar in control and

423   smDKO strips (Fig. 3D). These results may suggest that L-type Ca^{2+} channel-dependent

424   contraction involves both C2α/C2β-dependent and -independent contractile

425   mechanisms whereas L-type Ca^{2+} channel-independent contraction does not require

426   C2α or C2β. Y-27632 partially inhibited spontaneous- and oxytocin-induced

427   contraction in control strips, but did not significantly inhibit contraction of smDKO

428   strips (Fig. 3E), suggesting that both Rho kinase-dependent and -independent contractile

429   mechanisms are involved in spontaneous and oxytocin-induced contraction in control

430   strips. In contrast, contraction of smDKO uterine smooth muscle is largely Rho

431   kinase-independent. It is suggested that Rho kinase-dependent contraction requires C2α

432   and C2β whereas Rho kinase-independent one does not.

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433

Oxytocin-induced phosphorylation of MLC_{20} and MYPT1 is attenuated in uterine

smooth muscle tissues of smDKO mice

We compared oxytocin-induced phosphorylation of MLC_{20} and MYPT1, the latter

phosphorylation of which inhibits MLC_{20} phosphatase to lead to an increase in MLC_{20}

phosphorylation, in uterine strips of smDKO and control mice. Oxytocin induced a

1.6-fold increase in mono-phosphorylation (Ser^{19}) of MLC_{20} (p-MLC_{20}) in uterine

strips of control mice, but not in those of smDKO mice (Fig. 4A). Oxytocin also

induced a 2.6-fold increase in di-phosphorylation (Thr^{18}/Ser^{19}) of MLC_{20} (pp-MLC_{20}),

which is known to be increased when MLC_{20} phosphatase is inhibited or MLCK

activity becomes very high (27) , in control mouse uterine strips (Fig. 4B). In contrast,

in smDKO mouse strips, oxytocin did not increase pp-MLC_{20} level above that in the

non-stimulated control strips. Furthermore, oxytocin induced a 2.0-fold increase in

phosphorylation (Thr^{853}) of the myosin light chain$_{20}$ phosphatase (MLCP)-regulatory

subunit MYPT1, which results in inhibition of MLCP, in control mouse strips but not in

smDKO mouse strips (Fig. 4C). Thus, both mono- and di-phosphorylation levels of

MLC_{20} are reduced with diminished inhibition of MLCP in smDKO mouse strips.

450

Rho signaling but not Ca²⁺ signaling is downregulated in smDKO myometrial cells

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452   We isolated myometrial cells from control and smDKO pregnant mice. The

453   morphology of the isolated cells observed under bright field microscopy was similar

454   between control and smDKO mice (Fig. 5A). We identified 70 % of the isolated cells as

455   smooth muscle, using mice that expressed td-Tomato reporter gene specifically in

456   smooth muscle (Fig. 5B).

457   We determined oxytocin-induced changes in the $[Ca^{2+}]i$ in isolated myometrial

458   smooth muscle. Oxytocin induced similar extents (approximately 6-fold) of the peak

459   increases in the $[Ca^{2+}]i$ in both control and smDKO myometrial smooth muscle (Fig.

460   5C). Because uterine smooth muscle contraction is dependent on L-type Ca^{2+} channels,

461   we analyzed the protein expression of the α_2 subunit ($Cav_{\alpha2}$) of L-type calcium channel

462   in control and smDKO mice uterus. The expression level of $Cav_{\alpha2}$ protein in the uterus

463   was similar in control and smDKO mice as evaluated by Western blotting (Fig. 5D).

464   Immunofluorescence of the uterine tissues with anti-$Cav_{\alpha2}$ antibody showed that the

465   myometrial smooth muscle in both control and smDKO uterus expressed this protein

466   (Fig. 5E, left). With SRRF microscopy, we obtained higher resolution views of the

467   immunostained myometrial layer, which showed that $Cav_{\alpha2}$ was distributed mainly on

468   the plasma membrane or its vicinity of smooth muscle (Fig. 5E left). BK channel, which

469   is a negative regulator of L-type calcium channels, was also distributed largely on the

470   plasma membrane or its vicinity of smooth muscle, and there was no detectable

471   difference of BK channel expression in control and smDKO mouse uterus (Fig. 5E

472   right).

473   The phosphorylation of MYPT1, which is controlled mainly by Rho kinase

474   downstream of Rho, was decreased in smDKO uterine smooth muscle (Fig. 4C).

475   Therefore, we determined oxytocin-induced Rho activation in isolated myometrial

476   smooth muscle cells, using a FRET imaging technique. In control smooth muscle cells,

477   we observed substantial oxytocin-induced Rho activation signals at the plasma

478   membrane and the intracellular compartment. In smDKO myometrial smooth muscle

479   cells, Rho activation signals at both the plasma membrane and the intracellular

480   compartment were diminished (Fig. 5F).

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Discussion

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488   In parturition, the uterine smooth muscle develops potent contractile force to expel

489   the fetus. Dramatic changes in the signaling and contractile machineries of uterine

490   smooth muscle as well as the neuroendocrine functions at the end of pregnancy allows

491   generation of potent uterine contraction at parturition (1,2,28,29) . In the present study,

492   we identified the novel smooth muscle molecules, class II PI3K C2α and C2β, which

493   are required for full contraction of uterine smooth muscle and normal delivery. C2α and

494   C2β are involved in activation of Rho and resultant Rho-kinase-dependent inhibition of

495   MLCP, which potentiates MLC_{20} phosphorylation and contractile force in uterine

496   smooth muscle (27-31) . Highly homologous C2α and C2β completely compensate for

497   the effects of single gene deletion of each other in parturition, and only double KO

498   results in defects in parturition.

499   In uterine smooth muscle, activation of cell surface receptors including oxytocin and

500   prostaglandin $F_{2\alpha}$ and Ca^{2+} channels including the L-type voltage-dependent Ca^{2+}

501   channels triggers an increase in $[Ca^{2+}]_i$ by Ca^{2+} release from the intracellular Ca^{2+} store

502   and Ca^{2+} entry through the plasma membrane Ca^{2+} channels (28,30,32) . The increase in

503   the $[Ca^{2+}]i$ activates Ca^{2+}/calmodulin-dependent MLCK (Ca^{2+}-MLCK pathway),

504   leading to MLC_{20} phosphorylation and smooth muscle contraction. Oxytocin and

28

505   prostaglandin $F_{2\alpha}$ also activate small GTPase Rho via the heterotrimeric G protein

506     $G_{12/13}$ (28,30,31). Rho activates its effector Rho kinase, resulting in inhibition of MLCP

507   by phosphorylating the regulatory subunit of MLCP, MYPT1 (Rho-Rho kinase-MLCP

508   pathway) (27). Inhibition of MLCP potentiates MLCK-catalyzed MLC_{20}

509   phosphorylation and contraction. Therefore, Rho-MLCP pathway as well as

510   Ca^{2+}-MLCK pathway coordinately and effectively increase MLC_{20} phosphorylation,

511   thus playing a critical role in oxytocin-induced uterine smooth muscle contraction

512   (28-31,33,34) . The Ca^{2+} signaling pathway and Rho-Rho kinase signaling pathways

513   may have cross-communication because it was reported that Ca^{2+} entry across the

514   plasma membrane activated Rho and Rho kinase in several different types of smooth

515   muscle and that Rho kinase activated voltage-dependent Ca^{2+} channels through

516   phosphorylation (22,23,35) . Importantly, the uterine smooth muscle in the late

517   pregnancy has increased oxytocin receptor number and the depolarization of the resting

518   membrane potential, which increase excitability and contraction of uterine smooth

519   muscle (28,29,31-34).

520   The contraction data in the present study showed that not only oxytocin-induced

521   contraction but also spontaneous and KCl membrane depolarization-induced contraction

522   was attenuated in smDKO uterine smooth muscle compared with control smooth muscle.

523 Mechanistically, spontaneous and membrane depolarization-induced contraction as well

524 as oxytocin-induced contraction were inhibited by a Rho kinase inhibitor, suggesting the

525 contribution of Rho kinase pathway to these contractile responses in control mice.

526 Notably, the extents of contraction inhibition by a Rho kinase inhibitor were marginal in

527 smDKO uterine smooth muscle, suggesting that the contribution of Rho kinase pathway

528 to contraction was greatly diminished in smDKO smooth muscle. The decreased

529 MYPT1 phosphorylation and MLC_{20} di-phosphorylation in oxytocin-stimulated smooth

530 muscle supported this notion (27). The direct measurements of Rho activation in the

531 myometrial cells by the FRET imaging technique showed that oxytocin-induced Rho

532 activation was decreased in smDKO myometrial cells. Thus, it is likely that the

533 decreased Rho activity results in a higher MLCP activity, leading to reductions in

534 MLC_{20} phosphorylation and contraction in smDKO smooth muscle. These data also

535 suggest in smDKO smooth muscle that decreased Rho kinase activity might induce

536 diminished activation of L-type Ca^{2+} channels and, thereby, reduced spontaneous and

537 KCl-induced contraction (35).

538   In our previous study (14), we found that VEGF-induced Rho activation occurred in

539 the intracellular endosomal compartment, in which the internalized VEGF receptor-2

540 resided in endothelial cells. Both processes of the VEGF receptor-2 internalization and

541   endosomal Rho activation required C2α. We also observed that C2α is required for the

542   endosomal signaling induced by other receptor ligands including S1P and TGFβ1 (9,15).

543   The catalytic product of C2α, $PI(3,4)P_2$, contributes to endocytosis by recruiting various

544     $PI(3,4)P_2$-binding domain-containing proteins, which include SNX9, and promoting

545   clathrin-coated vesicle formation (36) . A role of C2β in endocytosis is currently almost

546   unknown. Since C2α single KO did not impair uterine smooth muscle contraction, very

547   likely C2β can compensate for a defect caused by C2α deficiency in uterine smooth

548   muscle. In smDKO uterine smooth muscle, the amount of $PI(3,4)P_2$ produced by

549   slightly expressed C2α and C2β is probably insufficient for receptor internalization and

550   subsequent signaling in the endosomes in which receptors and their associated signaling

551   molecules are assembled. A special form of Rho-guanine nucleotide exchange factor

552   (Rho-GEF) is implicated in Rho activation in the endosomes (37,38). The Rho-GEF,

553   Syx, which was found to be involved in VEGF-induced regulation of cell junctions, is

554   one of the candidate Rho-GEFs. Futher studies are required for identifying a Rho-GEF

555   involved in endosomal Rho activation and clarifying the mechanism of recruitment and

556   activation of a Rho-GEF.

557   Because RhoA-Rho kinase-MLCP pathway is a major contractile mechanism in

558   spontaneous and uterotonin-induced contraction (28), the new role of these two class II

559 PI3K isoforms in Rho-Rho kinase-MLCP pathway may provide some insight about the

560 mechanisms for clinical conditions of abnormal uterine contraction including preterm

561 labor, uterine inertia and life-threatening postpartum hemorrhage. For preterm labor,

562 tocolytic agents are administered (39). Administration of class II PI3K inhibitors, which

563 inhibit Rho and thereby stimulate MLCP in uterine smooth muscle, may be candidates

564 for developing new tocolytic agents. On the contrary, for clinical conditions of

565 insufficient uterine contraction such as uterine inertia and life-threatening postpartum

566 hemorrhage, stimulators of class II PI3Ks may bring about beneficial outcome by

567 increasing Rho activity and thereby inhibiting MLCP activity with augmented uterine

568 contraction. The inhibitory and stimulatory mechanisms of C2α and C2β at cellular

569 levels are currently not well understood. Therefore, further studies are required to

570 unravel the regulatory mechanism of human class II PI3K activity.

571   In summary, our study showed the importance of the novel molecules, C2α and

572 C2β, in the regulation of the Rho-Rho kinase-MLCP pathway and their requirement for

573 full contraction of uterine smooth muscle and parturition. It could be possible that

574 dysfunctions of class II PI3K might lead to clinical conditions caused by abnormal

575 uterine smooth muscle contraction. Further studies are required to explore the detailed

576 mechanisms of class II PI3K actions and the regulation of their activity, which provides

577   further insight into the physiological and pathophysiological roles of class II PI3Ks.

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580   **Acknowledgements**

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721 **Figure Legends**

722 **Figure 1.** Impaired pup delivery in smooth muscle-specific C2α- and C2β-double KO

723 mice. (**A**) Immunofluorescent staining of myosin heavy chain 11 (Mhc11) in the smooth

724 muscle layer of uterus and bladder in SM22α-Cre;ROSA26-tdTomato reporter mice.

725 Mhc11-positive cells express tdTomato protein. Schematic for the generation of mice

726 with smooth muscle-specific expression of tdTomato fluorescent protein is shown on

727 the top. (**B**) Immunofluorescent staining of C2α and C2β in the uterine myometrium of

728 control and smDKO pregnant mice at gestational day (GD) 18.5. M, myometrium. EM,

729 endometrium. (**C**) Time-course of the pup delivery from control and smDKO female

730 mice. (**D**) The uteri of pregnant mice and the numbers of pups within the uteri at GD

731 18.5. The magnified views of the boxed portion of the uteri in the most left pictures are

732 shown in (**i**) and (**ii**). In (**C**) and (**D**), the numbers in the blanket denote the numbers of

733 analyzed pregnant mice. The data in Fig. 1C and D are expressed as means $\pm$ SE. * p

734 < 0.05.

735

736 **Figure 2.** No abnormality in uterine morphology and smooth muscle-specific protein

737 expression in control and C2α- and C2β-double KO mice. (**A**) Gross views of the uteri

738 of control and smDKO non-pregnant mice. (**B**) HE staining of uterine sections of

739   control and smDKO non-pregnant mice and myometrial thickness of control and

740   smDKO mice. The representative histological images (left) and quantified data (right).

741   (**C**) Expression of various smooth muscle-specific proteins in the myometrial tissues of

742   non-pregnant and pregnant (GD18.5) control and smDKO mice (top). The

743   representative western blots (top) and quantified data (bottom). (**D**) Expression of C2α

744   and C2β proteins in the myometrial tissues of non-pregnant and pregnant (GD18.5)

745   control and smDKO mice (top). The representative western blots (top) and quantified

746   data (bottom). In (**B**) and (**D**), the numbers in the blanket denote the numbers of

747   analyzed mice. The data in Fig. 2B, C and D are expressed as means $\pm$ SE.   * p <

748   0.05 and ** p < 0.01.

749

750   **Figure 3.** Diminished contractile responses with reduced Rho kinase dependence in

751   uterine smooth muscle of C2α- and C2β-double KO mice. The isometric tension was

752   determined in the uterine strips from control, single smKO (smC2αKO and smC2βKO)

753   mice, and smDKO mice. The amplitudes and frequency of spontaneous contraction (**A**),

754   KCl-induced contraction (**B**), and oxytocin-induced contraction (**C**). The uterine strips

755   were stimulated with 200 nM oxytocin or 60 mM KCl or unstimulated, and maximal

756   tensions and spike frequency were determined. (**D**) Reductions of contraction by the

42

757   L-type Ca^{2+} channel antagonist nitrendipine (NTD). The amplitudes and frequency of

758   spontaneous contraction and amplitudes of oxytocin-induced contraction were

759   determined with or without NTD (100 nM) pretreatment as described in "Materials and

760   Methods". (**E**) Reductions of contraction by the Rho kinase inhibitor Y27632. The

761   amplitudes and frequency of spontaneous contraction and amplitudes of

762   oxytocin-induced contraction were determined with or without Y27632 (10 μM)

763   pretreatment as described in "Materials and Methods". In (**A**) to (**E**), the numbers in the

764   blanket denote the numbers of analyzed strips. The data are expressed as means $\pm$ SE.

765   * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$.

766

767   **Figure 4.** Diminished oxytocin-induced phosphorylation of 20 kDa myosin light chain

768   and MYPT1 in uterine smooth muscle of C2α- and C2β-double KO mice.

769   The uterine smooth muscle strips isometrically contracted in response to oxytocin (200

770   nM) stimulation were snap-frozen and analyzed for phosphorylation of MLC_{20} at Ser^{19}

771   (p-MLC_{20}) (**A**) and at Thr^{18}/Ser^{19} (pp-MLC_{20}) (**B**), and MYPT1 (p-MYPT1) at Thr^{853}

772   (**C**). The uterine smooth muscle strips were frozen at 3, 10 and 10 min, respectively,

773   after oxytocin addition for the determinations of p-MLC_{20}, pp-MLC_{20}, and p-MYPT1.

774   In (**A**) to (**C**), the numbers in the blanket denote the numbers of analyzed samples. The

775   data are expressed as means $\pm$ SE. * $p < 0.05$ and ** $p < 0.01$.

776

777   **Figure 5.** Diminished oxytocin-induced Rho activation, but not intracellular Ca^{2+}

778   mobilization or Ca^{2+} channel protein expression, in myometrial smooth muscle cells

779   from C2α- and C2β-DKO mice. Myometrial smooth muscle cells were isolated from

780   control and DKO mice. (**A**) The phase-contrast images of myometrial smooth muscle

781   cells. (**B**) Smooth muscle-specific expression of tdTomato fluorescent protein in mice

782   that carries R26-tdTomato reporter construct. Nuclei were stained with DAPI. (**C**)

783   Oxytocin-induced increase in the $[Ca^{2+}]i$ in uterine smooth muscle cells from control

784   and smDKO mice. Cells were stimulated with 100 nM oxytocin. The quantified data

785   show the $[Ca^{2+}]i$ peak response from 21 control and 20 smDKO cells. (**D**) Western

786   blotting of L-type Ca^{2+} channel protein $Cav_{\alpha2}$ in uterine smooth muscle cells from

787   control and smDKO mice. (**E**) Immunofluorescent staining of the Ca^{2+} channel $Cav_{\alpha2}$

788   and the K^+ channel BKα1 in the myometrium of control and smDKO mice. The boxed

789   regions were shown as the magnified views obtained with SRRF microscopy, "M",

790   myometrial layer of uterus. The red arrowheads denote $Cav_{\alpha2}$ protein of L-type Ca^{2+}

791   channel in the first SRRF view panel (left) and BKα1 protein of K^+ channel of last

792   SRRF view panel (right). (**F**) FRET imaging of Rho activation in uterine smooth muscle

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793   cells from control and smDKO mice. Left, representative images of Rho-FRET signals.

794   Right, quantified data from 9 control and 9 smDKO cells. The peak/basal signal ratio in

795   control cells were expressed as 100%. Red arrowhead denotes rho activation signal. In

796   (**C**), (**D**) to (**F**), the numbers in the blanket denote the numbers of analyzed samples. The

797   data in Fig. 5C, D and F are expressed as means $\pm$ SE. ***$p < 0.001$.

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A SM22α-Cre ; R26^tdTomato

B

Control (female): C2α^fl/fl; C2β^fl/fl

smDKO (female): C2α^fl/fl; C2β^fl/fl; SM22α-Cre

C

D

Fig. 1

A
Control   smDKO
5 mm

B
Control   smDKO
500 μm   500 μm
i   ii
M   M
20 μm   20 μm

ns

Myometrial thickness (μm)

Control   smDKO
(5)   (5)

C
Non-pregnant   Pregnant

Mhc11 ►
MLCK ►
Caldesmon ►
Calponin ►
PP1δ ►
MYPT1 ►
αSMA ►

Control   smDKO   Control   smDKO

■ Control (n = 3)
□ smDKO (n = 3)

Relative protein expression (%) (normalized to αSMA)

Mhc11   MLCK   Caldesmon   Calponin   PP1δ   MYPT1

Non-pregnant / Pregnant

D
Non-pregnant   Pregnant

PI3K-C2α ►
PI3K-C2β ►
αSMA ►

Control   smDKO   Control   smDKO

■ Control
■ smDKO

Relative protein expression (%) (normalized to αSMA)

PI3K-C2α   PI3K-C2β

**   *   *   *ns*

Non-pregnant   Pregnant   Non-pregnant   Pregnant
(3)   (3)   (3)   (3)   (3)   (3)   (3)   (3)

Fig. 2

Fig. 3

A

p-MLC$_{20}$

Total MLC$_{20}$

αSMA

Cont smDKO Cont smDKO

Oxytocin : − − + +

■ Control ☐ smDKO

Relative p-MLC$_{20}$/MLC$_{20}$ ratio
(-fold increase over (-) control)

2.0
1.5
1.0
0.5
0.0

**

**

(3) (3) (3) (3)

Oxytocin: − +

B

pp-MLC$_{20}$

Total MLC$_{20}$

αSMA

Cont smDKO Cont smDKO

Oxytocin : − − + +

■ Control ☐ smDKO

Relative pp-MLC$_{20}$/MLC$_{20}$ ratio
(-fold increase over (-) control)

4
3
2
1
0

*

*

(3) (3) (3) (3)

Oxytocin: − +

C

p-MYPT1

Total MYPT1

αSMA

Cont smDKO Cont smDKO

Oxytocin : − − + +

■ Control ☐ smDKO

Relative p-MYPT1/MYPT1 ratio
(-fold increase over (-) control)

2.5
2.0
1.5
1.0
0.5
0.0

*

*

(3) (3) (3) (3)

Oxytocin: − +

Fig. 4

A

Control   smDKO
DIC   DIC
100 μm   100 μm

B

Control   smDKO
tdTomato / DAPI   tdTomato / DAPI
10 μm   10 μm

R26-tdTomato

C

Control   Oxy   smDKO   Oxy

[Ca²⁺]ᵢ Fluo-8 F/F0 ratio (-fold increases over baseline)

Time (sec)   Time (sec)

[Ca²⁺]ᵢ peak response (F/Fo) (-fold increases over baseline)

ns

Control (21)   smDKO (20)

D

Control   smDKO

Cavα2
αSMA

Relative Cavα2 level (%) (Normalized to α-SMA)

ns

Control (3)   smDKO (3)

E

Cavα2 / DAPI   BKα1 / DAPI

Control   Magnified SRRF view   Control   Magnified SRRF view
M   20 μm   5 μm   M   20 μm   5 μm

smDKO   Magnified SRRF view   smDKO   Magnified SRRF view
M   20 μm   5 μm   20 μm   5 μm

F

Oxy   0 min   1 min   2 min   3 min

Control
Rho FRET
FRET ratio: High Low
20 μm

smDKO
Rho FRET

RhoA FRET peak response (F/F0)

***

Control (9)   smDKO (9)

Fig. 5