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TRADD, TRAF2, RIP1 and TAK1 are required for TNF- α -induced pro-labour mediators in human primary myometrial cells

Running Title: TNF- α signalling in human myometrium

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1 ABSTRACT

2

3 Problem: TNF- α plays a central role in the processes of human labour and delivery. This study
4 sought to determine the role of the adaptor proteins TNFR1-associated death domain protein
5 (TRADD), TNF receptor-associated factor 2 (TRAF2), receptor interacting protein 1 (RIP1) and
6 transforming growth factor beta-activated kinase 1 (TAK1) in TNF- α -induced formation of pro-
7 labour mediators.

8 Method of Study: Human primary myometrial cells were transfected with siRNA against TRADD
9 (siTRADD), TRAF2 (siTRAF2), RIP1 (siRIP1) or TAK1 (siTAK1), treated with TNF- α , and
10 assayed for pro-inflammatory mediators expression.

11 Results: siTRADD, siTRAF2, siRIP1 and siTAK1 significantly decreased TNF- α -induced IL-1 α ,
12 IL-1 β , IL-6, IL-8, MCP-1 mRNA expression and release of IL-6, IL-8 and MCP-1; and
13 cyclooxygenase (COX)-2 expression and release of prostaglandin PGF_{2 α} . There was a significant
14 attenuation of TNF- α -induced expression of adhesion molecules ICAM-1 and VCAM-1 mRNA
15 with siTRADD, siTRAF2 or siRIP1. siTRADD and siRIP1 significantly attenuated TNF- α -induced
16 MMP-9 mRNA expression and release and nuclear factor κ B (NF- κ B) transcriptional activity.
17 There was a significant increase in TNF- α -induced sVCAM-1 release, MMP-9 mRNA expression
18 and NF- κ B activity with siTAK1.

19 Conclusions: TRADD, TRAF2, RIP1 and TAK1 are involved in TNF- α signalling in human
20 myometrium. Further studies are required to determine if inhibition of these proteins can prevent
21 preterm birth.

22

23 INTRODUCTION

24

25 Preterm birth occurs in approximately 10% of all births worldwide ¹. The ensuing prematurity of the
26 newborns is the biggest contributing factor to perinatal morbidity and mortality ². Many children
27 born preterm will suffer life-long neurological and developmental problems. In addition to
28 emotional stress on families faced with preterm birth and having a newborn in intensive care, the
29 financial costs for individuals and health care systems are enormous. There are no therapeutics that
30 can reduce the high incidence of preterm birth and prevent its adverse outcomes. Thus, a greater
31 understanding of the mechanisms regulating term and preterm parturition are required.

32

33 TNF- α , released from leukocytes invading the intrauterine cavity during parturition ³, plays an
34 important role in the processes of human labour and delivery. Studies in humans demonstrated
35 higher amniotic fluid concentrations of TNF- α in patients who subsequently delivered preterm than

36 in patients who delivered at term ⁴. Likewise, high concentrations of TNF- α in the vaginal or
37 cervical secretions in women with symptoms of preterm labour are associated with early preterm
38 delivery ⁵. Elevated TNF- α levels are also detected in amniotic fluid during pregnancies
39 complicated by infection and preterm delivery in humans ⁶. In non-human primates, injection of the
40 amniotic cavity with TNF- α induces preterm labour ⁷. *In vivo*, intraamniotic infusion of TNF- α is
41 associated with significant elevations in pro-inflammatory cytokines, chemokines, prostaglandins,
42 MMP-9 and leukocytes in amniotic fluid ⁷. Likewise, *in vitro*, TNF- α can amplify or initiate the
43 process of parturition by further increasing cytokine production ⁸; promoting the synthesis of
44 cyclooxygenase (COX)-2, prostaglandin F_{2 α} (PGF_{2 α}), and the PGF_{2 α} receptor (FP) ⁹⁻¹¹ which are
45 important regulators of myometrial contractility; activating the extracellular matrix remodelling
46 enzyme matrix metalloproteinase (MMP)-9 ¹², and induce the expression of adhesion molecules ¹¹
47 that can further recruit leukocytes into the uterus. Therefore, understanding the intermediates
48 involved in the TNF- α signalling pathway is critical for the development of therapeutics to manage
49 or prevent preterm birth.

50

51 The canonical (or classical) NF- κ B pathway has a major role in the control of innate immunity and
52 inflammation ¹³. The NF- κ B pathway also plays a central role in the terminal processes of human
53 labour and delivery ¹⁴. In human gestational tissue, NF- κ B is activated by a broad range of stimuli
54 including TNF- α . Following stimulation, I κ B- α , which typically holds the NF- κ B RelA-p50
55 heterodimer in the cytoplasm, is phosphorylated by I κ B kinase (IKK), leading to its rapid
56 degradation. This allows free NF- κ B to translocate to the nucleus where it binds to κ B sites, leading
57 to gene transcription. In human myometrium, NF- κ B has been shown to control the transcription of
58 a number of pro-labour genes including IL-6, IL-8, COX-2 and MMP-9.

59

60 The adaptor proteins TNFR1-associated death domain protein (TRADD), TNF receptor-associated
61 factor 2 (TRAF2), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1 and cIAP2), receptor
62 interacting protein 1 (RIP1) and transforming growth factor beta-activated kinase 1 (TAK1) have
63 emerged as critical signalling intermediates of TNF- α -induced inflammation ¹⁵⁻¹⁸. TNF- α binds to
64 its cell surface receptor TNFR1 which leads to the recruitment of the intracellular death domain
65 (DD)-containing adaptor TRADD. Recruitment of TRADD can i) promote the association of the
66 TNFR1 complex with Fas-associated DD (FADD) which induces caspase activation and cell death;
67 or ii) recruit TRAF2, RIP1, cIAPs and TAK1, which trigger NF- κ B activation and resultant pro-
68 inflammatory responses ¹⁹.

69

70 We have previously shown cIAP1 and cIAP2 are required for TNF- α -induced expression of pro-
71 labour mediators in human myometrium⁹. The role, however, of TRADD, TRAF2, RIP1 and
72 TAK1 in the genesis of pro-inflammatory and pro-labour mediators induced by TNF- α in
73 myometrium is not known. Thus, the aim of this study was to use siRNA to determine if TRADD,
74 TRAF2, RIP1 and TAK1 are involved in TNF- α -induced expression and secretion of pro-
75 inflammatory cytokines; COX-2 expression and subsequent prostaglandin release; expression and
76 secretion of adhesion molecules ICAM-1 and VCAM-1; and expression of the ECM remodelling
77 enzyme MMP-9 in primary human myometrial cells.

78

79 **MATERIALS AND METHODS**

80

81 **Tissue collection**

82 The Research Ethics Committee of Mercy Hospital for Women approved this study. Written,
83 informed consent was obtained from all participating women. All myometrial samples were
84 obtained from women who delivered healthy, singleton infants at term (37-41 weeks gestation)
85 undergoing elective Caesarean section in the absence of labour. All tissues were brought to the
86 research laboratory and processed within 15 mins of the Caesarean delivery. Women with any
87 underlying medical conditions such as diabetes, asthma, polycystic ovary syndrome, preeclampsia
88 and macrovascular complications were excluded. Additionally, women with multiple pregnancies,
89 obese women, and fetuses with chromosomal abnormalities were excluded.

90

91 **Primary myometrial cell culture**

92 Cells were isolated and cultured as previously described¹¹. Briefly, myometrium was minced and
93 digested for 1 h in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-
94 12) with 3 mg/ml type 1 collagenase (Worthington Biochemical, Freehold, USA) and 80 μ g/ml
95 DNase 1 (Roche Diagnostics, Castle Hill, Australia) at 37°C. Cells were centrifuged at 400 \times g for
96 10 min and grown in DMEM/F-12 enriched with 10% heat-inactivated FCS (containing 100 U/ml
97 penicillin G and 100 mg/ml streptomycin).

98

99 **TRADD, TRAF2, RIP1 and TAK1 siRNA transfection**

100 Transfection of primary myometrial cells was performed as we have previously described¹¹.
101 Briefly, cells at approximately 50% confluence were transfected using Lipofectamine 3000
102 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). TRADD
103 siRNA (siTRADD), TRAF2 siRNA (siTRAF2), TAK1 siRNA (siTAK1) and negative control
104 siRNA (siCONT) were obtained from Ambion (Thermo Fisher Scientific; Scoresby, Vic, Australia).

105 RIP1 siRNA (siRIP1) was obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology,
106 Santa Cruz, CA, USA). Cells were transfected with 50 nM siRNA (TRADD, TRAF2 or TAK1) or
107 100 nM siRNA (RIP1) in DMEM/F-12 for 48 h followed by treatment with or without 10 ng/ml
108 TNF- α (PeproTech; Rocky Hill, NJ, USA) for 24 h. Cells were collected and stored at -80°C until
109 assayed for mRNA expression by qRT-PCR as detailed below. Media was collected and stored at
110 -80°C until assayed for cytokine and prostaglandin release as detailed below. Cell viability was
111 assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)
112 proliferation assay as we have previously described [11](#). The response to TNF- α between patients
113 varied greatly, as we have previously reported [11](#). Thus, data is presented as fold change in
114 expression relative to the expression level in TNF- α -stimulated siCONT transfected cells, which
115 was set at 1. Experiments were performed from myometrium obtained from five patients.

116

117 **NF- κ B luciferase assay**

118 A luciferase assay was also used to determine the possible interactions between TRADD, TRAF2,
119 RIP1 and NF- κ B as previously described [11](#). Primary myometrial cells were transfected with 300
120 ng/ml NF- κ B reporter construct (Qiagen; Chadstone Centre, Vic, Australia) using FuGENE HD
121 transfection reagent (Promega; Alexandria NSW, Australia). After 6 h, cells were transfected with
122 50 nM siRNA (TRADD, TRAF2 or TAK1) or 100 nM of siRIP1 or siCONT (as detailed above) for
123 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA), with or without 10
124 ng/ml TNF- α and the cells incubated at 37°C for an additional 20 h. After final incubation, cells
125 were harvested in lysis buffer, and luminescence activity was measured using a Luciferase Reporter
126 Assay Kit (Life Research; Scoresby, Vic, Australia) and Renilla Luciferase Flash Assay kit
127 (Thermo Fisher Scientific; Scoresby, Vic, Australia) as instructed. The ratio of the firefly luciferase
128 level to the Renilla luciferase level was determined and the results are expressed as a ratio of
129 normalised luciferase activity. The experiments were performed from myometrium obtained from
130 five patients.

131

132 **RNA extraction and qRT-PCR**

133 RNA extractions and qRT-PCR was performed as previously described [11](#). RNA quality and
134 integrity were measured using a NanoDrop ND1000 and determined via the A_{260}/A_{280} ratio. RNA
135 (0.2 μg) was converted to cDNA using the high-capacity cDNA reverse transcription kit according
136 to the manufacturer's instructions (Applied Biosystems; Waltham, MA, USA). RT-PCR was
137 performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville,
138 NSW, Australia) using 100 nM of pre-designed and validated QuantiTect primers (primer
139 sequences not available) (Qiagen; Chadstone Centre, Vic, Australia). Average gene Ct values were

140 normalised against two housekeeping genes (β 2-Microglobulin (B2M) and succinate dehydrogenase
141 complex subunit A (SDHA)). Fold differences were determined using the comparative Ct method.

142

143 **Enzyme immunoassays**

144 Assessment of cytokine and chemokine release of IL-6 and IL-8 was performed using the
145 CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies;
146 Mulgrave, Vic, Australia). The release of MCP-1, sICAM-1 and sVCAM-1 was performed by
147 sandwich ELISA from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's
148 instructions. The release of PGF_{2α} into the incubation medium was assayed using a commercially
149 available competitive enzyme immunoassay kit according to the manufacturer's specifications
150 (Kookaburra Kits from Sapphire Bioscience, NSW, Australia). The interassay and intraassay
151 coefficients of variation for all assays were less than 10%.

152

153 **Gelatin zymography**

154 Incubation media was also collected and assessment of MMP-9 was performed by gelatin
155 zymography as previously described ²⁰. Proteolytic activity was visualized as clear zones of lysis on
156 a blue background of undigested gelatin. Gels were scanned using a ChemiDoc XRS system (Bio-
157 Rad Laboratories; Gladesville, NSW, Australia), inverted, and densitometry performed using **using**
158 Quantity One image analysis software (Bio-Rad Laboratories; Gladesville, NSW, Australia). Fold
159 change was calculated relative to TNF- α , which was set at 1.

160

161 **Statistical analysis**

162 All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA,
163 USA). The homogeneity of data was assessed by the Bartlett's test, and when significant, the data
164 were logarithmically transformed before further analysis using a one-way ANOVA (with LSD post-
165 hoc testing to discriminate among the means). Statistical significance was ascribed to a *P* value
166 <0.05. Data were expressed as mean \pm SEM.

167

168 **RESULTS**

169

170 **Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on pro-inflammatory cytokines and** 171 **chemokines**

172 The efficacy of siRNA transfection is demonstrated in Supplementary Figure 1. When compared to
173 siCONT transfected cells, there was ~85% decrease in TRADD mRNA with siTRADD; 60%
174 decrease in TRAF2 mRNA expression with siTRAF2; 70% decrease in RIP1 mRNA expression

175 with siRIP1; and 90% decrease in TAK1 mRNA expression with siTAK1. A MTT cell viability
176 assay showed no difference in absorbance between cells transfected with siCONT or siTRADD,
177 siTRAF2, siRIP1 or siTAK1 (Supplementary Figure 1).

178
179 For subsequent experiments, after siRNA transfection, cells were treated with TNF- α . As expected,
180 in siCONT cells, TNF- α significantly increased IL-6, IL-8 and MCP-1 mRNA
181 expression and secretion (Figure 1). This increase was significantly decreased in siTRADD (Figures
182 1A-F), siTRAF2 (Figures 1A-F), siRIP1 (Figures 1G-L) or siTAK1 (Figures 1M-R) transfected
183 cells. In addition, IL-1 α and IL-1 β mRNA expression was also increased by TNF- α treatment in
184 siCONT transfected cells. The effect of siTRADD, siTRAF2, siRIP1 or siTAK1 was a significant
185 decrease in IL-1 α and IL-1 β mRNA expression (Table I). As we have previously reported, IL-1 α
186 and IL-1 β levels are not detectable in the incubation media from human primary myometrial cells ¹⁰
187 and thus not assessed.

188

189 **Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on COX-2-prostaglandin pathway**

190 Treatment of primary myometrial cells with TNF- α significantly increased COX-2 (Figures
191 2A,D,G) and FP (Figures 2B,E,H) mRNA expression, and subsequent PGF_{2 α} release (Figures
192 2C,F,I). The effect of siTRADD (Figures 2A-C), siTRAF2 (Figures 2A-C), siRIP (Figures 2D-F) or
193 siTAK1 (Figures 2G-I) was a significantly attenuation of TNF- α -induced COX-2 and FP mRNA
194 expression and PGF_{2 α} release.

195

196 **Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on adhesion molecules**

197 As expected, ICAM-1 and VCAM-1 mRNA expression, and sICAM-1 and sVCAM-1 were
198 significantly increased in siCONT cells incubated with TNF- α (Figure 3). TNF- α -induced ICAM-1
199 mRNA expression and sICAM-1 release was significantly attenuated in cells transfected with
200 siTRADD (Figures 3A,B), siTRAF2 (Figures 3A,B), siRIP1 (Figures 3E,F), or siTAK1 (Figures
201 3I,J). TNF- α -induced VCAM-1 mRNA expression was significantly suppressed by siTRADD
202 (Figures 3C), siTRAF2 (Figures 3C), siRIP1 (Figures 3G), or siTAK1 (Figures 3K). Further, TNF-
203 α -induced sVCAM-1 release was also significantly decreased by siTRADD (Figure 3D) or siRIP1
204 (Figure 3H). There was no effect of siTRAF2 on TNF- α -induced sVCAM-1 release (Figure 3D),
205 while siTAK1 significantly augmented TNF- α -induced sVCAM-1 release (Figure 3L).

206

207 **Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on MMP-9**

208 Figure 4 demonstrates the effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression of
209 the ECM degrading and remodelling enzyme MMP-9. In siCONT transfected cells, TNF- α

210 significantly increased MMP-9 mRNA expression (Figures 4A,C,E) and pro MMP-9 secretion
211 (Figures 4B,D,F). The effect of siTRADD (Figure 4A), siTRAF2 (Figure 4A) or siRIP1 (Figure 4C)
212 was a significant suppression of TNF- α -induced MMP-9 mRNA. In addition, siTRADD (Figure 4B
213 and siRIP1 (Figure 4D) also significantly reduced TNF- α -induced pro MMP-9 secretion. There
214 was, however, no effect of siTRAF2 on pro MMP-9 secretion (Figure 4B). Unexpectedly, siTAK1
215 significantly augmented MMP-9 mRNA expression (Figure 4E) and pro MMP-9 secretion (Figure
216 4F).

217

218

219 **Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on NF- κ B activation**

220 The final aim of this study was to investigate if TRADD, TRAF2, RIP1 and TAK1 elicits their
221 effects via interfering with NF- κ B activation. As expected, TNF- α significantly increased NF- κ B
222 transcriptional activity (Figure 5). The result of siTRADD (Figure 5A) and siRIP1 (Figure 5C) was
223 a significant suppression of TNF- α induced NF- κ B activation. On the other hand, there was no
224 effect of siTRAF2 on TNF- α induced NF- κ B activation (Figure 5B) while siTAK1 further
225 augmented TNF- α induced NF- κ B activation (Figure 5D).

226

227 **DISCUSSION**

228

229 The novel findings of this study are that in human myometrium, TRADD, TRAF2, RIP1 and TAK1
230 are required for TNF- α -induced expression of pro-labour mediators. Specifically, loss-of-function
231 studies demonstrated that in the absence of TRADD, TRAF2, RIP1 and TAK1, the expression and
232 or secretion of pro-inflammatory cytokines, chemokines, prostanoids and cell adhesion molecules to
233 TNF- α was, for the most part, significantly blunted. These findings are in concordance with studies
234 in non-gestational tissues [15-18](#).

235

236 TNF- α exists in two biologically active forms, a membrane-bound form (mTNF- α) and a soluble
237 form (sTNF- α), which bind to their cognate receptors TNFR1 and TNFR2. Engagement of TNFR1
238 rapidly leads to the assembly of a membrane associated protein complex (termed complex I)
239 consisting of TRADD, TRAF2 and RIP1. Activation of TNFR1 recruits the adapter protein
240 TRADD and the serine–threonine kinase RIP1. TRADD can further recruit the adapter protein
241 TRAF2 via its N-terminal TRAF-binding domain. Complex 1 can activate numerous signalling
242 pathways to induce inflammation, activate both pro-apoptotic and anti-apoptotic pathways or
243 promote cell growth [21](#). Pro-inflammatory signalling, however, is the most favoured pathway
244 through the recruitment of TAK1 and the IKK complex resulting in NF- κ B activation. Complex 1

245 also activates activator protein (AP)-1 and mitogen-activated protein kinase (MAPK) signalling by
246 recruitment of TAK1.

247

248 In this study, knockdown of TRADD, TRAF2, RIP1 or TAK1 in primary myometrial cells
249 significantly suppressed TNF- α -induced expression and secretion of pro-inflammatory cytokines
250 and chemokines. These findings are of significance given the central role of pro-inflammatory
251 cytokines and chemokines in the processes of labour and delivery, both at term and preterm⁸.
252 Concomitant with increased macrophage and neutrophil infiltrate³, myometrium of healthy
253 labouring women is associated with an upregulation of mRNA expression of numerous pro-
254 inflammatory cytokines (e.g. IL-1 β , IL-6, TNF- α) and chemokines (e.g. IL-8, MCP-1)²². Pro-
255 inflammatory cytokines can then further induce: (i) cytokine release through positive feed forward
256 mechanism; (ii) the expression of adhesion molecules; (iii) COX-2 expression and subsequent
257 prostaglandin production, and (iv) ECM matrix remodelling enzymes such as MMP-9. Collectively,
258 these events facilitate human labour and delivery.

259

260 Prostaglandins play an important role in the regulation of parturition. COX-2 is the rate limiting
261 enzyme involved in the conversion of arachidonic acid into prostaglandins. Increased expression of
262 COX-2 observed in myometrium before labour onset²³ is responsible for the increased synthesis of
263 prostaglandins observed during labour. PGF_{2 α} is an important mediator of uterine contractility²⁴,
264 exerting its actions via its receptor, FP²⁵ whose expression is also increased at term labour in
265 myometrium²⁶. TNF- α has been shown to induce the expression of COX-2 and FP⁹⁻¹¹ and
266 stimulate the synthesis of prostanoids^{9, 10} in human myometrial cells. In this study, we show that
267 TRADD, TRAF2, RIP1 or TAK1 are required for TNF- α -induced COX-2 and FP mRNA
268 expression and resultant prostaglandin secretion.

269

270 The expression of the cell adhesion molecules such as ICAM-1 and VCAM-1 are increased in
271 human cervix and myometrium during pregnancy and parturition^{27, 28}; in part due to the expression
272 of infiltrating leukocytes in the myometrium, cervix and fetal membranes at parturition^{3, 29}. These
273 adhesion molecules can then attract neutrophils, macrophages and T cells to invade these tissues.
274 Cytokines, induced by stretch, play an essential role in leukocyte migration³⁰. We have previously
275 shown that TNF- α can induce ICAM-1, VCAM-1 and MMP-9 mRNA expression and secretion
276 from human myometrial cells¹¹. TNF- α can also induce the expression of MMP-9^{11, 31} which is
277 involved in tissue remodelling of human myometrium during labour. In this study, we demonstrated
278 a requirement for TRADD, TRAF2 and RIP1 in TNF- α -induced ICAM-1, VCAM-1 and MMP-9
279 expression and/or secretion in primary myometrial cells. Of note, while VCAM-1 and MMP-9

280 mRNA expression were decreased by siTRAF2, the secretion of sVCAM-1 and pro MMP-9 were
281 unchanged; possibly due to post-translation modifications. Furthermore, and in contrast to
282 siTRADD, siTRAF2 or siRIP1, siTAK1 significantly augmented MMP-9 mRNA expression and
283 pro MMP-9 secretion. The reason for the increase in MMP-9 is not known; but **may** it may be due
284 to activation of pathways downstream of TAK1 (discussed below).

285
286 Mice with genetic knockout of TRADD, TRAF2, RIP1 or TAK1 have clearly demonstrated their
287 roles in TNF- α -induced NF- κ B signalling ^{17, 32-34}. *In vitro* studies, using siRNA, have confirmed the
288 role of these proteins in TNF- α signalling in a numbers of different cells. For example, TRADD,
289 TRAF2 or TAK1 are required for TNF- α -induced NF- κ B activation in mouse embryonic fibroblasts
290 (MEFs) and RIP1-deficient Jurkat T cells are specifically defective in the activation of NF- κ B in
291 response to TNF- α stimulation ¹⁵⁻¹⁸. In support, this study reports that TRADD and RIP1 are
292 required for TNF- α -induced NF- κ B signalling in human myometrial cells. On the other hand, there
293 was no effect of siTRAF2 on NF- κ B transcriptional activity while NF- κ B activity was increased by
294 siTAK1. TRAF2 ³⁵ and TAK1 ³⁶, in addition to NF- κ B, can also transduce signals to c-Jun N-
295 terminal kinase (JNK) and or p38 mitogen-activated protein kinase (p38 MAPK) of the mitogen-
296 activated protein kinase (MAPK) pathway. Ultimately, other transcription factors downstream of
297 p38 MAPK and JNK are activated including c-Jun and ATF2, resulting in the transcription of genes
298 important for inflammatory and immune responses. p38 MAPK is involved in the regulation of pro-
299 inflammatory and pro-labour mediators in fetal membranes ³⁷ while specific inhibitors of JNK
300 delays preterm labour and decreases inflammation in a mouse model of preterm birth ³⁸. It is thus
301 possible that TRAF2 and TAK1 regulate pro-inflammatory and pro-labour mediators in human
302 myometrial cells via the p38 MAPK and/or JNK signalling pathways. Alternatively, only one time
303 point was analysed in this study; it is possible that longer or shorter incubations are required with
304 TNF- α to see an effect on NF- κ B transcriptional activity.

305 **An avenue of further research, is the effect of siTRADD, siTRAF2, siRIP1 or siTAK1 on TNF- α -**
306 **induced uterine contractions. The myometrium is maintained in a relatively quiescent state during**
307 **pregnancy but is transformed into a contractile state at term labour. TNF- α has been shown to**
308 **enhance contractions in human primary uterine smooth muscle cells ³⁹. A number of proteins,**
309 **termed contractions associated proteins (CAPs), have been shown to be important in this process,**
310 **including FP. Given that we showed that TRADD, TRAF2, RIP1 or TAK1 are required for TNF- α -**
311 **induced FP mRNA expression, it would also be of interest to determine if they play a role in**
312 **regulating myometrial contractions in response to TNF- α treatment.**

313

314 In conclusion, for the first time, we report that TRADD, TRAF2, RIP1 and TAK1 play a role in the
315 regulating TNF- α signalling in human myometrium. These findings are of significance given the
316 central role of TNF- α in the processes of human labour and delivery. Further studies are required to
317 elucidate the significance of TRADD, TRAF2, RIP1 and TAK1 in human labour and delivery, and
318 whether inhibition of these proteins may be able to prevent preterm birth. Notably, inhibition of
319 TAK1 has been reported to block inflammation in sheep. Specifically, intra-amniotic treatment with
320 the TAK1 inhibitor 5z-7-oxozeaeno reduced amniotic fluid levels of PGE₂ and fetal membrane
321 leukocyte infiltration induced by the bacterial product LPS ⁴⁰.

322

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327

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333

334 **DISCLOSURE SUMMARY**

335 The authors have nothing to declare.

336

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Table I. Effect of siTRADD, siTRAF2, siRIP and siTAK1 on IL-1 α and IL-1 β mRNA expression

	IL-1 α mRNA expression	IL-1 β mRNA expression
siCONT	0.04 \pm 0.02*	0.00 \pm 0.00*
siCONT + TNF- α	1.00 \pm 0.00	1.00 \pm 0.00
siTRADD + TNF- α	0.50 \pm 0.16*	0.12 \pm 0.03*
siTRAF2 + TNF- α	0.50 \pm 0.10*	0.03 \pm 0.02*
siCONT	0.02 \pm 0.00*	0.00 \pm 0.00*

siCONT + TNF- α	1.00 \pm 0.00	1.00 \pm 0.00
siRIP1 + TNF- α	0.41 \pm 0.07*	0.28 \pm 0.12*
siCONT	0.05 \pm 0.03*	0.03 \pm 0.01*
siCONT + TNF- α	1.00 \pm 0.00	1.00 \pm 0.00
siTAK1 + TNF- α	0.23 \pm 0.09*	0.09 \pm 0.03*

For all data, the fold change was calculated relative to TNF- α -stimulated siCONT transfected cells, and displayed as mean \pm SEM.

* P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

FIGURE LEGENDS

Figure 1. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on pro-inflammatory cytokines and chemokines.

Human primary myometrial cells were transfected with (A-F) 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, (G-L) 100 nM siRIP1 or 100 nM siCONT, or (M-R) 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF- α for an additional 24 h (n=5 patients).

(A,C,E,G,I,K,M,O,Q) IL-6, IL-8 and MCP-1 mRNA expression was analysed by qRT-PCR.

(B,D,F,H,J,L,N,P,R) IL-6, IL-8 and MCP-1 concentration in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to TNF- α -stimulated siCONT transfected cells, and displayed as mean \pm SEM. * P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

Figure 2. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the COX-2-prostaglandin pathway.

Human primary myometrial cells were transfected with (A-C) 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, (D-F) 100 nM siRIP1 or 100 nM siCONT, or (G-I) 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF- α for an additional 24 h (n=5 patients).

(A,B,D,E,G,H) COX-2 and FP mRNA expression was analysed by qRT-PCR. (C,F,I) PGF_{2 α}

concentration in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to TNF- α -stimulated siCONT transfected cells, and displayed as mean \pm SEM.

* P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

Figure 3. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression and secretion of adhesion molecules.

Human primary myometrial cells were transfected with (A-D) 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, (E-H) 100 nM siRIP1 or 100 nM siCONT, or (I-L) 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF- α for an additional 24 h (n=5 patients). (A,C,E,G,I,K) ICAM-1 and VCAM-1 mRNA expression was analysed by qRT-PCR. (B,D,F,H,J,L) sICAM-1 and sVCAM-1 concentrations in the incubation media was assayed by ELISA. For all data, the fold change was calculated relative to TNF- α -stimulated siCONT transfected cells and data displayed as mean \pm SEM. * P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

Figure 4. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression and secretion of MMP-9 expression.

Human primary myometrial cells were transfected with (A,B) 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, (C,D) 100 nM siRIP1 or 100 nM siCONT, or (E,F) 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF- α for an additional 24 h (n=5 patients). (A,C,E) MMP-9 mRNA expression was analysed by qRT-PCR. (B,D,F) The incubation medium was assayed for pro MMP-9 expression by gelatin zymography. Representative zymography image from 1 patient is shown. For all data, the fold change was calculated relative to TNF- α -stimulated siCONT transfected cells and data displayed as mean \pm SEM. * P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

Figure 5. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on NF- κ B activation.

Human myometrial cells were transfected with 300 ng/ml NF- κ B reporter construct. After 6 h, cells were transfected with (A) 50 nM siTRADD or 50 nM siCONT, (B) 50 nM siTRAF2 or 50 nM siCONT, (C) 100 nM siRIP1 or 100 nM siCONT, or (D) 50 nM siTAK1 or 50 nM siCONT for 48 h, then treated with 10 ng/ml TNF- α for an additional 20 h (n=5 patients). Promoter activity is expressed as a ratio of luciferase activity of TNF- α stimulated siCONT transfected cells. All data displayed as mean \pm SEM. * P <0.05 vs. TNF- α -stimulated siCONT transfected cells (one-way ANOVA).

Figure 1

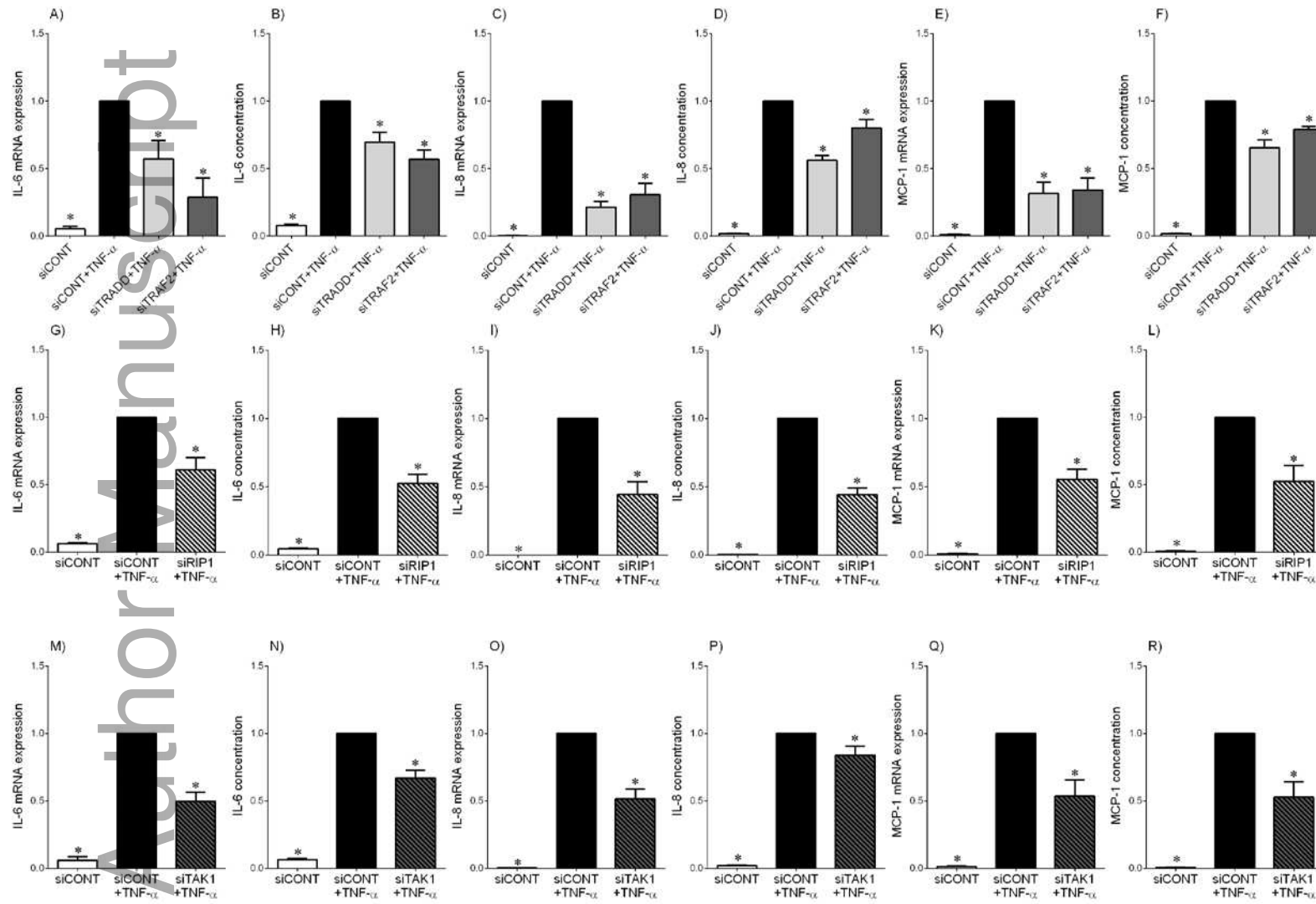


Figure 2

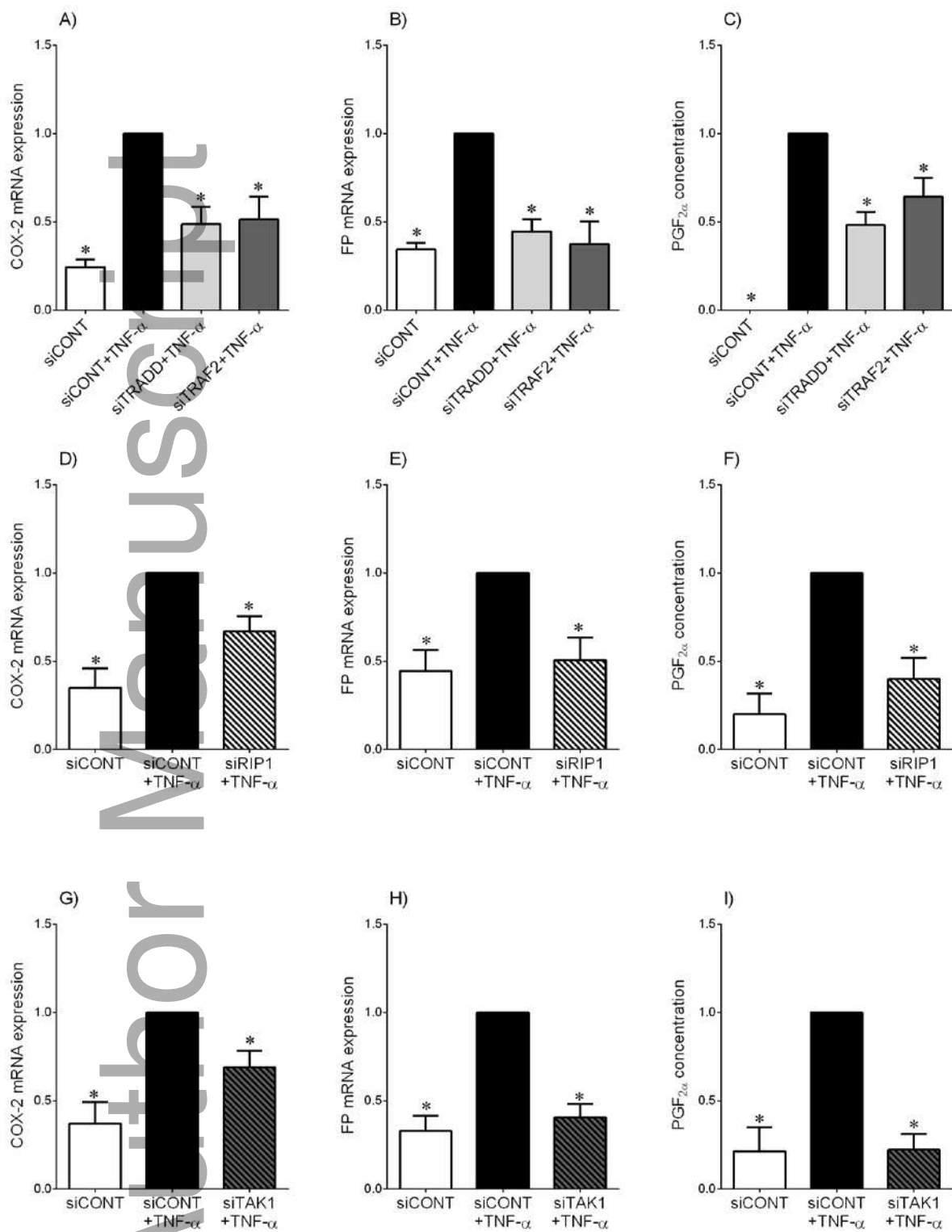


Figure 3

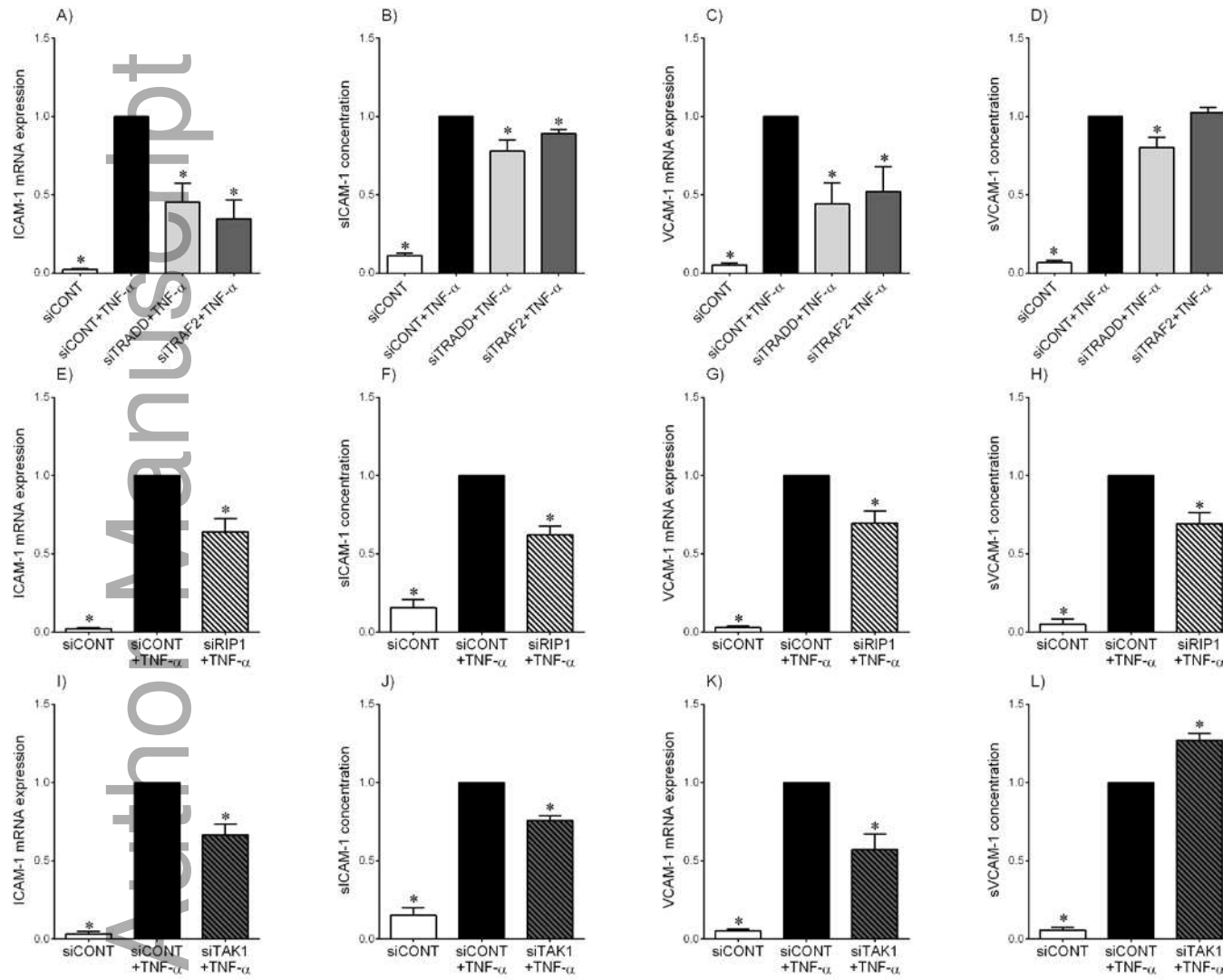


Figure 4

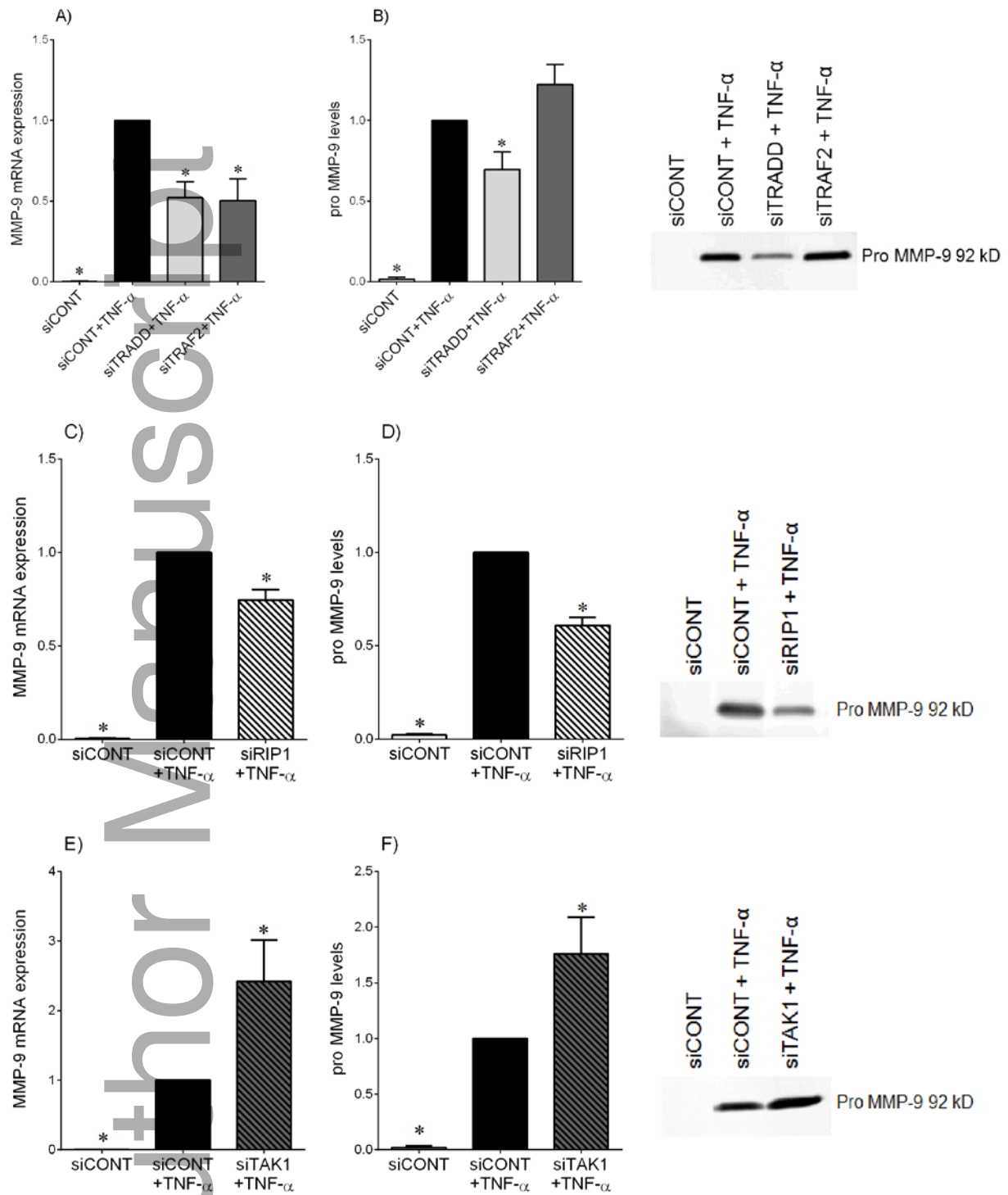
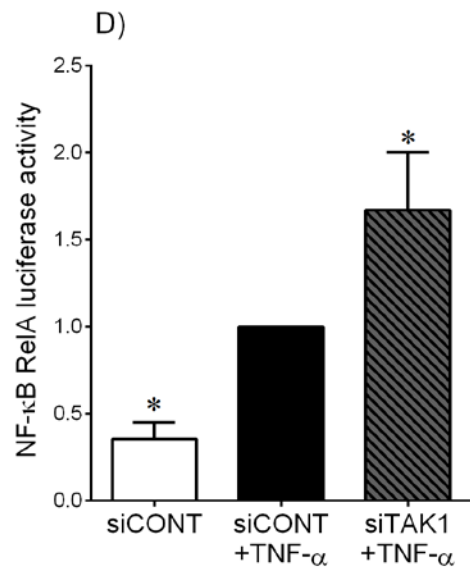
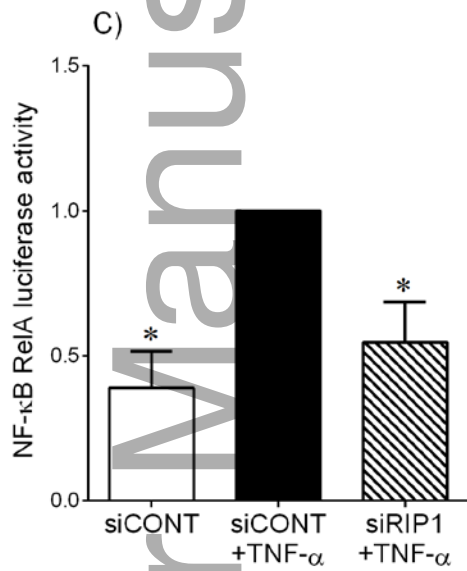
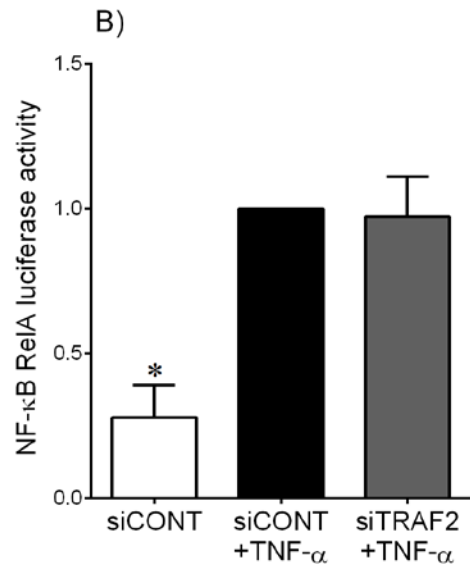
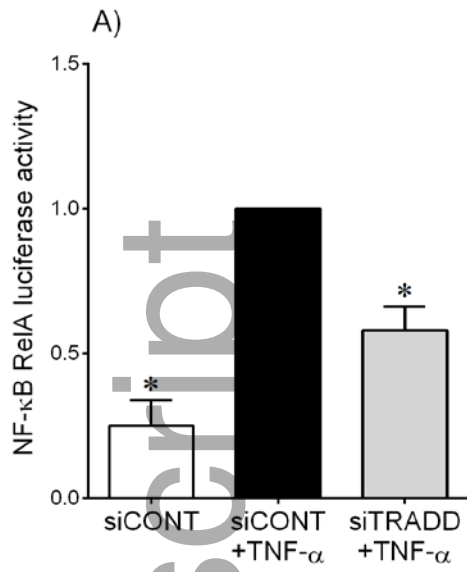


Figure 5



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