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Citation for published version:

Young, VJ, Ahmad, SF, Brown, JK, Duncan, WC & Horne, AW 2016, 'ID2 mediates the transforming growth factor-1-induced Warburg-like effect seen in the peritoneum of women with endometriosis', *Molecular Human Reproduction*. <https://doi.org/10.1093/molehr/gaw045>

Digital Object Identifier (DOI):

[10.1093/molehr/gaw045](https://doi.org/10.1093/molehr/gaw045)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Molecular Human Reproduction

Publisher Rights Statement:

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Vicky J Young; Syed F Ahmad; Jeremy K Brown; W Colin Duncan; Andrew W Horne, *Molecular Human Reproduction* 2016;

doi: 10.1093/molehr/gaw045

in *Molecular Human Reproduction* following peer review. The definitive publisher-authenticated version is available online at:

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ID2 mediates the Transforming Growth Factor- β 1-induced Warburg-like effect seen in the peritoneum of women with endometriosis

Journal:	<i>Molecular Human Reproduction</i>
Manuscript ID	MHR-15-0284.R2
Manuscript Type:	Original Research
Date Submitted by the Author:	13-Jun-2016
Complete List of Authors:	Young, Vicky Ahmad, Syed; University of Edinburgh, MRC Centre for Reproductive Health Brown, Jeremy Duncan, Colin Horne, Andrew; University of Edinburgh, MRC Centre for Reproductive Health
Key Words:	Lactate, HIF-1 α , Inhibitor of DNA binding protein 2, Mesothelium, endometriosis

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 Manuscripts

1 **ID2 mediates the transforming growth factor- β 1-induced Warburg-like effect**
2 **seen in the peritoneum of women with endometriosis**

3

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12 **Running title:**

13 ID2 and TGF- β 1 regulate metabolism in endometriosis

14

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24

25 **Abstract**

26

27 **Study question:** Is inhibitor of DNA binding protein 2 (ID2) a mediator of the
28 transforming growth factor (TGF)- β 1-induced Warburg-like effect seen in the
29 peritoneum of women with endometriosis?

30 **Study finding:** The TGF- β 1-induced changes in the metabolic phenotype of
31 peritoneal mesothelial cells from women with endometriosis are mediated through the
32 ID2 pathway.

33 **What is known already:** TGF- β 1 induces the metabolic conversion of glucose to
34 lactate via aerobic glycolysis (the 'Warburg effect') in the peritoneum of women with
35 endometriosis, through increased expression of the transcription factor hypoxia
36 inducible factor α (HIF-1 α). ID proteins are transcriptional targets of TGF- β 1.

37 **Study design, samples/materials, and methods:** Expression of ID2 was investigated
38 in luteal phase peritoneal biopsies from women with regular menstrual cycles, with
39 and without endometriosis (n=8-10 each group) by quantitative RT-PCR (qRT-PCR)
40 and immunohistochemistry. *ID2* mRNA expression in primary human peritoneal
41 mesothelial cells (HPMC) and immortalized mesothelial cells (MeT-5A) was assessed
42 by qRT-PCR (n=6). The effects of TGF- β 1 and ID2 siRNA on HIF-1 α mRNA
43 expression and lactate secretion was assessed using qRT-PCR and a colorimetric
44 lactate assay.

45 **Main results and the role of chance:** ID2 is localised to peritoneal mesothelial and
46 stromal cells of women with and without endometriosis. *ID2* mRNA expression is
47 lower in peritoneum adjacent to the endometriosis lesions compared to distal sites
48 (p<0.01). Exposure of HPMC and MeT-5A cells to physiological concentrations of
49 TGF- β 1 decreases *ID2* mRNA expression (P<0.01, P<0.001, respectively, versus

50 control). ID2 knockdown increases HIF-1 α mRNA expression ($p < 0.01$) and lactate
51 secretion ($p < 0.05$ versus scrambled control) to the same degree as with exposure to
52 TGF- β 1.

53 **Limitations, reasons for caution:** Primary human cell cultures and a cell line were
54 used in this study, and thus the results may not fully represent the situation *in vivo*.
55 The results should also be replicated using a larger number of samples.

56 **Wider implications of the findings:** Novel therapeutics that target the TGF β /ID
57 pathway offer a potential role in the treatment of endometriosis.

58 **Large scale data:** N/A

59 **Study funding and competing interest(s):** This work was funded by a Wellbeing of
60 Women research grant (R42533) awarded to AWH, JKB and WCD; and an MRC
61 Centre Grant G1002033. VJY received grant support from Federation of Women
62 Graduates (134225) and a PhD studentship from the College of Medicine and
63 Veterinary Medicine at the University of Edinburgh. There are no competing interests
64 to declare.

65 **Key Words:**

66 endometriosis, lactate, hypoxia inducible factor-1 α , inhibitor of DNA binding protein
67 2, mesothelium

68

69 **Introduction**

70

71 Endometriosis is a benign inflammatory disorder, defined by the presence of
72 endometrial tissue outside the uterus with lesions typically found on the pelvic
73 peritoneum in close association with the peritoneal mesothelium (Giudice, 2010). The
74 prevalence of endometriosis is estimated at 2-10% of women of reproductive age and
75 it is associated with chronic pelvic pain, dysmenorrhoea, dyspareunia and infertility
76 (Giudice, 2010). Surgical excision can provide symptom relief, but symptoms recur in
77 up to 75% of surgical cases and available medical treatments have undesirable side
78 effects and are contraceptive (Jacobson, et al., 2009). The aetiology of endometriosis
79 is uncertain. However, increasing evidence suggests endometriosis lesions may share
80 characteristics with tumours (including resistance to apoptosis, angiogenesis and
81 invasion) and changes in the peritoneal microenvironment are thought to contribute to
82 the pathophysiology of this disease (Jacobson, Duffy, Barlow, Koninckx and Garry,
83 2009, Young, et al., 2014, Young, et al., 2013).

84

85 Aberrant expression of transforming growth factor- β 1 (TGF- β 1) is well defined
86 during tumorigenesis and has been shown to induce 'the Warburg effect', the
87 metabolic conversion of glucose to lactate in normoxia (Fosslien, 2008). High levels
88 of energy rich lactate 'feed' cancer cells allowing them to survive while increasing
89 angiogenesis, local inflammation and resistance to apoptosis, further fuelling tumour
90 progression and metastasis (Hirschhaeuser, et al., 2011). The Warburg effect is
91 induced by inflammatory cytokines, including TGF- β 1, through induction of the
92 transcription factor hypoxia inducible factor α (HIF-1 α) under normoxic conditions
93 (Fosslien, 2008). We have previously shown that endometriosis lesions and the

94 surrounding peritoneum use aerobic glycolysis as a means of energy production in a
95 similar fashion to the Warburg effect in tumorigenesis (Young, et al., 2014).
96 Furthermore, we found TGF- β 1 initiated Warburg-like metabolism, through induction
97 of HIF-1 α protein in aerobic conditions, in the peritoneal mesothelial cells. Increased
98 lactate levels, a by-product of glycolysis, were observed within the peritoneal fluid of
99 women with endometriosis (Young, et al., 2014). Overproduction of lactate increases
100 cell invasion, angiogenesis and immune suppression, all crucial steps in the
101 development of tumors and known regulators of endometriosis (Hirschhaeuser, Sattler
102 and Mueller-Klieser, 2011). Importantly, peritoneal fluid lactate levels directly
103 correlated with TGF- β 1 concentrations (Young, et al., 2014).

104

105 TGF- β 1 has been reported to regulate HIF-1 α mRNA and protein expression via the
106 inhibitor of DNA binding (ID) proteins in epithelial cells (Cao, et al., 2009). The IDs
107 are basic helix-loop-helix (bHLH) proteins that lack a DNA-binding domain but can
108 heterodimerise with other bHLH transcription factors, such as HIF-1 α , preventing
109 them from binding to DNA. This interaction can therefore have positive or negative
110 effects on gene expression (Ruzinova and Benezra, 2003). In particular, ID2 is
111 reported to be down-regulated by TGF- β signalling in epithelial cells and has been
112 shown to be involved in the TGF- β induced epithelial to mesenchymal transition and
113 increased tumour invasion (Kondo, et al., 2004). Much of this evidence is based on
114 decreased ID2 protein increasing HIF-1 α mRNA expression (Tsai and Wu, 2012) and
115 overexpression of ID2 has been shown to block HIF-1 α protein activation (Cao, et
116 al., 2009).

117

118 Here, we investigate the regulation and effects of ID2 expression in peritoneal
119 mesothelial cells. In particular, we investigate whether TGF- β 1 regulated ID2 may
120 account for increases in HIF-1 α expression and changes to peritoneal mesothelial cell
121 metabolism in women with endometriosis.
122

For Review Only

123 **Methods**

124

125 **Patients**

126 Ethical approval for this study was obtained from the Scotland A Research Ethics
127 Committee (LREC 11/AL/0376). Informed written consent was obtained from all
128 patients and all tissues were collected according to EPHeCt guidelines (Fassbender, et
129 al., 2014) and as previously described (Young, et al., 2014). Tissue biopsies were
130 collected from women with peritoneal (no evidence of ovarian or deep disease)
131 endometriosis (who had macroscopic evidence of endometriosis at laparoscopy and
132 where the diagnosis was confirmed by post-operative histological examination of the
133 lesions) and women without endometriosis (who displayed no evidence of
134 endometriosis or any other underlying pelvic pathology at laparoscopy). None of the
135 women were taking hormonal contraceptives at the time of tissue collection and they
136 all had regular 21-35 day menstrual cycles. All samples were collected in the luteal
137 phase of the cycle. In the women with endometriosis, we collected peritoneal
138 biopsies from peritoneum adjacent (1-3cm away from) (n=10) and distal (outside the
139 pelvic brim) (n=10) to endometriosis lesions. In the women without endometriosis,
140 we collected peritoneal biopsies from sites prone to endometriosis within the ovarian
141 fossae (n=8). Histological examination confirmed the absence of endometriosis
142 lesions in the peritoneal biopsies. Primary human peritoneal mesothelial cells
143 (HPMC) were collected at the time of surgery from women with and without
144 endometriosis as previously described (Young, et al., 2014) by gently brushing the
145 pelvic mesothelium with a TaoTM brush (QC Sciences, VA, USA).

146 Cell culture

147 HPMC were cultured in HOSE1 media containing; 40% media 199, 40% MCDB 105
148 and supplemented with 15% fetal bovine serum (FBS), 0.5% penicillin/streptomycin
149 and 1% L-glutamine, at 37°C under 5% CO₂ in air (Life Technologies Inc., Paisley
150 UK and Sigma Chemical Co., Poole UK). The MeT-5A mesothelial cell line (CRL-
151 9444, ATCC, Middlesex, UK) was cultured in Iscove's Modified Dulbecco's Media
152 (IMDM) supplemented with 10% FBS and 1% L-glutamine at 37°C under 5% CO₂ in
153 air (Life Technologies Inc.). HPMC and MeT-5A cells were plated at 1.5x10⁵ or
154 2x10⁵ cells/ml, respectively, in a 12 well plate and left to adhere for 12 hours before
155 being serum starved for 24 hours. Cells were exposed to physiological levels of
156 recombinant human TGF-β1 (2ng/ml) or, as mesothelial cells are a source of TGF-β1
157 (Young, et al., 2014), to a neutralising TGF-β antibody (0.5μg/ml) (R&D Systems,
158 Abingdon, UK) for between 3hr and 48hr.

159

160

161 siRNA knockdown

162 For siRNA knockdown, MeT-5A cells were plated at 3 x 10⁵ cells/well in a six-well
163 culture plate with ID2 siRNA (two different siRNA sequences were combined for
164 optimal knockdown) (Table 1) or scrambled siRNA (SincerR, Ambion) using the
165 siPORT neofection transfection kit (ThermoFisher Scientific, Loughborough, UK) for
166 48 hours. Successful transfection conditions were developed using positive control
167 GAPDH siRNA where reduced gene expression was confirmed at the mRNA level by
168 quantitative RT-PCR (qRT-PCR), at the protein level by Western blotting and
169 cytotoxicity was confirmed to be less than 15% using a lactate dehydrogenase assay

170 (Source Bioscience, Nottingham, UK) (data not shown). Successful ID2 knockdown
171 was confirmed by western blot and by qRT-PCR.

172

173 **Immunohistochemistry**

174 Peritoneal biopsies (n=3 in each clinical group) were examined by
175 immunohistochemistry, as previously described (Young, et al., 2014). Briefly,
176 sections of paraffin embedded tissue were mounted onto microscope slides and
177 dewaxed and rehydrated before antigen retrieval in 10mM Tris, 1mM EDTA pH 9
178 with 5 min of pressure-cooking. This was followed by incubation with 3% hydrogen
179 peroxide for 30 min and blocking in normal horse serum diluted 1:12 in Tris buffered
180 saline with 0.5% Tween 20 (TBST20) for 30min. Slides were incubated with primary
181 antibody overnight at 4°C (ID2 Santa Cruz sc-489 diluted 1:500, isotype match
182 control Rabbit IgG Dako X0903) and then washed in TBST20 before incubation with
183 species specific impress kit for 30 min at room temperature (Vector Laboratories,
184 Peterborough, UK). Slides were then washed and incubated with 3, 3'-
185 diaminobenzidine for 5min and counterstained with hematoxylin, dehydrated and
186 visualized by light microscopy, using an Olympus Provis microscope equipped with a
187 Kodak DCS330 camera (Olympus Optical Co., London, UK, and Kodak Ltd., Herts,
188 UK). Due to the limited supply of peritoneal tissue, both positive and negative
189 controls were performed on endometrial tissue.

190

191 **Immunoblotting**

192 Cell lysates (at a concentration of 200,000 cells/200µl, with 10µl loaded onto each
193 lane) were resolved on NuPAGE Novex 4-12% Bis-Tris polyacrylamide gels under
194 reducing conditions with NuPAGE MOPS sodium dodecyl sulphate running buffer

195 and according to the manufacturers' instructions (Life Technologies Inc.). Proteins
196 were transferred to a polyvinylidene difluoride membrane using a semi-dry blotter
197 and blocked with 5% milk powder in Tris-buffered saline with 0.1% Tween 20
198 (TBST-20). The membrane was incubated with mouse anti-GAPDH (Sigma G9545
199 0.5µg/ml) in TBS-T20 and 5% milk for 2 hours at room temperature, before
200 incubating with anti-ID2 (SantaCruz Sc-489, 1µg/ml) in TBS-T20 and 5% milk
201 overnight at 4°C. Following this membranes were washed and incubated with species
202 specific impress kit 1:10000 in TBS-T20 and 5% milk for 1 hour at room temperature
203 (Vector Laboratories Ltd., Peterborough, UK). The membrane was washed and
204 incubated with Tyramide for 30 minutes and imaged using a FujiFilm FLA-5100
205 Fluorescent Image Analyzer (PerkinElmer, Cambridgeshire, UK and Fujifilm Ltd.,
206 Bedford, UK).

207

208 **Transcript analysis**

209 RNA was isolated from all tissues/cells using the RNeasy Mini/maxi kit and cDNA
210 synthesis was performed using Superscript VILO Master Mix, according to
211 manufacturer's instructions (Qiagen, West Sussex, UK; Life Technologies).
212 Quantitative RT-PCR reactions were performed using brilliant III ultra-fast SYBR
213 green QPCR master mix with standard running conditions on an ABI Prism 7900 Fast
214 system (Agilent, Berkshire, UK, Applied Biosystems, Warrington, UK). Pre-validated
215 primers were used and melt curves were analyzed to confirm specific products (Table
216 2) (Primardesign, Southampton, UK). Messenger RNA transcripts were quantified
217 relative to the appropriate housekeeping gene *GAPDH* as determined by geNorm
218 assay and using the $2^{-\Delta Ct}$ or the $2^{-\Delta\Delta Ct}$ method.

219

220 Lactate assay

221 Lactate concentration in MeT-5A conditioned media was determined as previously
222 described (Young, et al., 2014) using a commercial kit adapted for use on a Cobas
223 Fara centrifugal analyzer (Roche Diagnostics Ltd., Welwyn Garden City, UK). The
224 linear range was 0.15 - 19 mmol/l, and the within and between batch coefficients of
225 variation were 0.9% and 2.7%, respectively.

226

227 Statistical analysis

228 All the results are from a minimum of three independent experiments expressed as
229 mean \pm SEM. Quantitative RT-PCR and lactate assay were analysed using students' *t*
230 tests or one-way ANOVA and Tukeys post-test to compare each group, generated
231 using GraphPad PRISM version 5 (GraphPad Software Inc., La Jolla, CA, USA)
232 statistical software and a *p* value of <0.05 was considered significant.

233

234 Results

235

236 HPMC express ID2

237 ID2 protein was localised to the mesothelial cells of the peritoneum from women with
238 and without endometriosis (Figure 1A). Endometrial tissue was used as positive
239 control and no staining was observed in the isotype-matched negative control (data
240 not shown). To investigate whether ID2 is differentially expressed in the peritoneum
241 of women with endometriosis, *ID2* expression was quantified by RT-PCR in
242 peritoneal biopsies from the sites adjacent and distal to endometriosis lesions from
243 women with endometriosis. We found that *ID2* expression was decreased in the
244 peritoneum adjacent to endometriosis lesions when compared to peritoneum distal to
245 endometriosis lesions ($P<0.05$; Figure 1B). In addition, both primary cultures of
246 HPMC and the peritoneal mesothelial cell line (MeT-5A) expressed *ID2* mRNA
247 (Figure 1C, D).

248

249 TGF- β 1 down-regulates *ID2* mRNA in HPMC

250 In order to assess the effect of TGF- β 1 on *ID2* expression in mesothelial cells,
251 HPMCs and MeT-5a cells were exposed to physiological concentrations of TGF- β 1
252 (2ng/ml). Exposure of HPMC to TGF- β 1 for 12 hours decreased *ID2* mRNA
253 expression ($P<0.01$ versus control; Figure 1D). Exposure of MeT-5A cells to TGF- β 1
254 for 3, 6, 12 and 24 hours showed that TGF- β 1 induced a rapid and sustained reduction
255 in *ID2* mRNA ($P<0.01$ - $P<0.001$ versus control; Figure 1E). Since mesothelial cells
256 are a source of TGF- β 1, for control purposes an inhibition of TGF- β activity by the anti-
257 TGF- β antibody was confirmed by immunostaining for phosphorylated Smad 2/3.
258 Immunostaining showed that treatment with anti-TGF- β antibody, at 0.5ug/ml as

259 recommended by the manufacturer, resulted in no positive staining of phosphorylated Smad
260 2/3 within the cell nucleus (Supplementary Figure 1).

261

262 **ID2 knockdown mimics TGF- β actions**

263 ID2 knockdown was confirmed at protein and mRNA level (Figures 2A and 2B,
264 respectively). *ID2* mRNA decreased after siRNA knockdown, in the presence or
265 absence of TGF- β 1 (Figure 2B). ID2 siRNA knockdown significantly increased HIF-
266 1 α mRNA expression in MeT-5A cells. (Figure 3A). ID2 siRNA knockdown also
267 significantly increased lactate production (Figure 3B). TGF- β 1 alone, or in
268 combination with ID2 siRNA, appeared to cause an increase in HIF-1 α mRNA and
269 lactate secretion (no statistical analysis). ID2 knockdown mimics the actions of TGF-
270 β 1 treatment as it upregulates HIF-1 α expression and lactate secretion, both in HPMC
271 and MeT-5a cells (Figure 3A, 3B).

272

273 **Discussion**

274

275 In this study, we demonstrated that ID2 protein is expressed in peritoneal mesothelial
276 cells from women with and without endometriosis and that *ID2* expression is
277 decreased in HPMC and MeT-5A cells upon exposure to physiological concentrations
278 of TGF- β 1. We also found that there is a local decrease in *ID2* expression in the
279 peritoneum adjacent to endometriosis lesions, consistent with locally increased TGF-
280 β 1 activity. Using siRNA, we demonstrate that decreasing levels of ID2 induce a
281 significant increase in HIF-1 α expression and lactate expression in MeT-5A cells,
282 similar to that seen with TGF- β 1.

283

284 We have previously shown that TGF- β 1 induces a change in metabolism from
285 oxidative phosphorylation to aerobic glycolysis in peritoneal mesothelial cells
286 adjacent to endometriosis lesions (Young, et al., 2014), a phenomenon which is
287 known as ‘Warburg Effect’ in tumorigenesis and has been shown to promote cell
288 invasion, angiogenesis and immune suppression (Vander Heiden, et al., 2009).
289 Interestingly, these are all crucial steps in the development and progression of
290 endometriosis. Our results presented here suggest that TGF- β 1 regulates changes in
291 metabolism in the peritoneal mesothelial cell through an ID2 - HIF-1 α pathway.

292

293 TGF- β 1 is a known regulator of *ID* gene expression in a variety of cells including
294 immune cells, endothelial cells and epithelial cells (Ruzinova and Benezra, 2003). In
295 epithelial cells, TGF- β , signalling through the Smad 2/3 pathway, classically inhibits
296 *ID2* by activating the transcriptional repressor ATF3 (activating transcription factor 3)
297 which in turns binds to the ATF/CREB (cAMP response element-binding protein) site

298 within the *ID2* promoter suppressing transcription (Kang, et al., 2003).
299 Overexpression of TGF- β 1 in breast cancer leads to a significant decrease in *ID2* and
300 this is linked to an increase in cell proliferation, invasion and extracellular matrix
301 (ECM) remodelling (Itahana, et al., 2003). Additionally, TGF- β 1 has been shown to
302 prevent apoptosis and induce proliferation in endometrial stromal cells (Rahimi and
303 Leof, 2007), to increase adhesion of normal human endometrial stromal cells to
304 mouse peritoneum (Beliard, et al., 2003) and to induce the invasion of ectopic
305 endometrial cells by facilitating the matrix metalloproteinases (MMPs) in the
306 peritoneal mesothelial cells via ECM remodelling in peritoneal adhesion formation
307 (Ma, et al., 1999). In the present study, we have shown that knockdown of *ID2* and
308 addition of TGF- β 1 induced lactate secretion from the mesothelial cells (i.e. a
309 Warburg-like effect). Of importance is that increased lactate secretion has been shown
310 to increase proliferation, adhesion and invasion in tumor cells and these are also the
311 key steps in the development and progression of endometriosis. Thus, increased
312 concentrations of TGF- β 1 in the peritoneal fluid and peritoneum of women with
313 endometriosis (Young, et. al., 2014) may decrease the expression of *ID2* in the
314 peritoneal mesothelial cells of women with endometriosis in a similar fashion.
315 Decreased *ID2* may increase bHLH transcription factors action and thus contribute to
316 lesion development by increasing the invasiveness of the peritoneal mesothelial cells
317 and inducing peritoneal ECM remodelling (Young, et al., 2013).

318

319 We also demonstrate that knockdown of *ID2* increases lactate secretion in peritoneal
320 mesothelial cells, suggesting that *ID2* may have effects on both HIF-1 α mRNA
321 expression and protein activation. This observation is supported by findings in
322 epithelial cells where overexpression of *ID2* blocked HIF-1 α activation by TGF- β

323 (Cao, et. al., 2009). In addition, we have previously shown that HIF-1 α mRNA is
324 increased in the peritoneum of women with endometriosis and in endometriosis
325 lesions, and this was attributed to increasing TGF- β 1 activity (Young, et al., 2014).
326 HIF-1 α is routinely degraded in the presence of oxygen through the ubiquitination
327 pathway, whereas hypoxia leads to HIF-1 α stabilisation and activity (Semenza,
328 2012). However HIF-1 α has previously been detected in normoxic conditions and
329 although the mechanism for this normoxic stabilisation remains unclear, it is thought
330 that increasing mRNA expression can overcome the protein degradation (Basu, et al.,
331 2011). In the current study we have shown that TGF- β regulates HIF-1 α at the
332 transcriptional level through decreased expression of ID2 under normoxic conditions.
333 This observation underpins a potential regulatory pathway for TGF- β induced HIF-1 α
334 expression and induction of a Warburg-like effect in endometriosis. Furthermore,
335 studies have shown HIF-1 α to regulate expression of ID2, indicating a potential
336 feedback cycle of expression (Lofstedt, et al., 2004).

337

338 TGF- β 1 has been shown to be upregulated in the peritoneal fluid from women with
339 endometriosis (Oosterlynck, et al., 1994, Young, et al., 2014). A recent study has
340 shown that TGF- β deficiency in the peritoneum of mice reduced lesion development
341 11-fold (Hull, et al., 2012). Moreover, the role of TGF- β in ovarian function has been
342 established and is emerging as a new therapeutic for manipulating ovarian function
343 and improving fertility (Knight and Glister, 2006). Thus, the TGF- β pathway may be
344 a potential fertility-sparing therapeutic target for endometriosis.

345

346 In conclusion, we present preliminary evidence that TGF- β 1 influences *ID2*
347 expression in peritoneal mesothelial cells. We also show here that *ID2* influences
348 HIF-1 α expression in peritoneal mesothelial cells. Taken together with our previous
349 findings showing that an increase in HIF-1 α increases local lactate production, these
350 results suggest that TGF- β 1 regulates changes in the metabolic phenotype of
351 peritoneal mesothelial cells via HIF-1A and through the *ID2* pathway. Increasing
352 lactate production by the peritoneum may facilitate ectopic endometrial cell survival
353 and invasion into the peritoneal mesothelium, promoting the development and
354 progression of endometriosis.

355

356 **Acknowledgements**

357 We are grateful to Prof Philippa Saunders for advice and guidance, Mrs Helen Dewart
358 and Mrs Ann Doust for patient recruitment and sample collection; Dr Forbes Howie
359 for assay development; Prof Steve Hillier for use of the MeT-5A cell line; Mr Bob
360 Morris, Mrs Frances Collins, Ms Arantza Esnal-Zufiurre and Mrs Jean Wade for
361 technical support; Mrs Sheila Milne for secretarial support and Mr Ronnie Grant and
362 Mr Jeremy Tavener for graphics support.

363

364 **Authors' roles**

365 AWH, WCD and VJY conceived and designed the project. VJY carried out the
366 laboratory work. VJY, SFA and JKB carried out the analysis. All authors contributed
367 to the manuscript write up.

368

369 **Funding**

370 This work was funded by a Wellbeing of Women research grant (R42533) awarded to
371 AWH, JKB and WCD. VJY receives grant support from Federation of Women
372 Graduates (134225) and a PhD studentship from the College of Medicine and
373 Veterinary Medicine at the University of Edinburgh. The data analysis and
374 preparation of this manuscript were supported by MRC Centre Grant G1002033.

375

376 **Conflict of interest**

377 The authors have no conflicts of interest.

378

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- 448
- 449

450 Figure legends

451

452 **Figure 1. Immunohistochemistry of paraffin embedded sections of human**
453 **peritoneum.**

454 Inhibitor of DNA binding protein 2 (ID2) protein expression is localised to the
455 mesothelial cells of peritoneum in women with (A) and without (B) endometriosis
456 (red arrows). Peritoneum adjacent to endometriosis lesions expressed significantly
457 lower levels of *ID2* mRNA when compared to peritoneum collected distal from
458 endometriosis lesions (C). Effect of transforming growth factor (TGF)- β 1 treatment
459 on *ID2* gene expression in human peritoneal mesothelial cells (HPMC) treated with
460 2ng/ml TGF- β 1 for 12 hours: TGF- β 1 treatment significantly decreased *ID2* mRNA
461 expression (** $p < 0.01$ versus control, $n = 6$) (D). Effect of TGF- β 1 treatment on *ID2*
462 gene expression in immortalized mesothelial (MeT-5A) cells treated with 2ng/ml
463 TGF- β 1 at different time points (E). TGF- β 1 treatment significantly decreased *ID2*
464 mRNA expression in the MeT-5A cells at 6, 12 and 24 hours after treatment when
465 compared to no treatment control cells at the same time point (data in D & E are
466 mean \pm SEM, ** $p < 0.01$, *** $p < 0.001$, $n = 3$). Scale bar: 20 μ m

467

468

469 **Figure 2. siRNA knockdown of ID2 in MeT-5A cells.**

470 MeT-5A cells were treated with siRNA for 48 hrs for gene expression studies. (A) A
471 representative western blot and summary graph ($n = 3$) of siRNA knockdown of ID2,
472 or scrambled siRNA control, and effect of TGF- β 1 on ID2 protein expression. (B)
473 siRNA knockdown of ID2 resulted in a significant decrease in *ID2* expression when

474 compared to scrambled siRNA in the MeT-5A cells. (data are mean \pm SEM,
475 $*p < 0.05$, $n = 3$).

476

477 **Figure 3. Effect of ID2 knockdown on HIF-1 α and lactate secretion in MeT-5A**
478 **cells.**

479 **(A)** ID2 siRNA significantly increased hypoxia inducible factor- 1 α (HIF-1 α) mRNA
480 expression in MeT-5A cells when compared to MeT-5A cells treated with scrambled
481 siRNA (mean \pm SEM, $*p < 0.05$, $n = 3$). **(B)** ID2 siRNA significantly increased lactate
482 concentrations in cell culture media from MeT-5A cells when compared to cell
483 culture media from MeT-5A cells treated with scrambled siRNA (mean \pm SEM,
484 $*p < 0.05$, $n = 3$). TGF- β 1 showed a non-significant increase in HIF-1 α mRNA
485 expression and lactate secretion, alone or in combination with ID2 siRNA.

486

487

488 **Supplementary Figure 1: Effect of anti-TGF- β 1 treatment on MeT-5A cells**
489 **exposed to TGF- β 1.**

490 Cells were pre-incubated with anti-TGF- β 1 followed by treatment with TGF- β 1
491 (2ng/ml) for 60 minutes and immunostained for pSmad 2/3. Cells treated with TGF-
492 β 1 only showed a noticeable increase in pSmad 2/3 expression and protein was seen
493 to relocate to the nucleus of cells when compared to cells blocked with anti-TGF- β 1
494 ($n = 3$). Scale Bar = 100 μ m

495

496

497

498

499 Table 1. siRNA oligonucleotide sequences used in a study of the roles of ID2 and
500 transforming growth factor- β 1 in endometriosis in women.

siRNA	Direction	Sequence 5' – 3'
ID2	Sense	GAAUCCCUUCUGAGUUAATT
	Anti-sense	UUAACUCAGAAGGGAAUUCAG
ID2	Sense	CGAUGAGCCUGCUAUACAATT
	Anti-sense	UUGUAUAGCAGGCUCAUCGGG

501 ID2: inhibitor of DNA binding protein 2

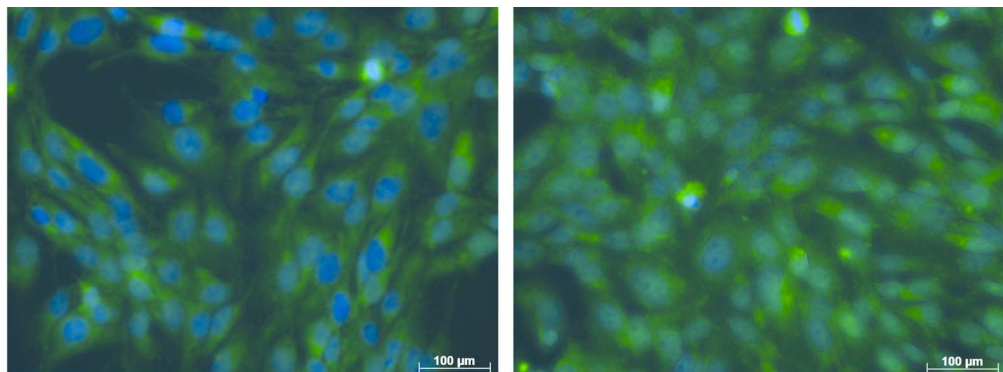
502 Table 2. Primers used for quantitative RT-PCR. All primers were pre-
503 validated and supplied by Primer Design.

Gene of interest	Direction	Primer sequence 5' to 3'
<i>ID2</i>	Sense	CGATGAGCCTGCTATAACA
	Anti-sense	AGGTCCAAGATGTAGTCGATGA
<i>HIF-1A</i>	Sense	TGCCACATCATCACCATATAGAG
	Anti-sense	TGACTCAAAGCGACAGATAACA

504 HIF-1 α : hypoxia inducible factor α

505

506



Anti TGF- β 1 +
TGF- β 1 +

-
+

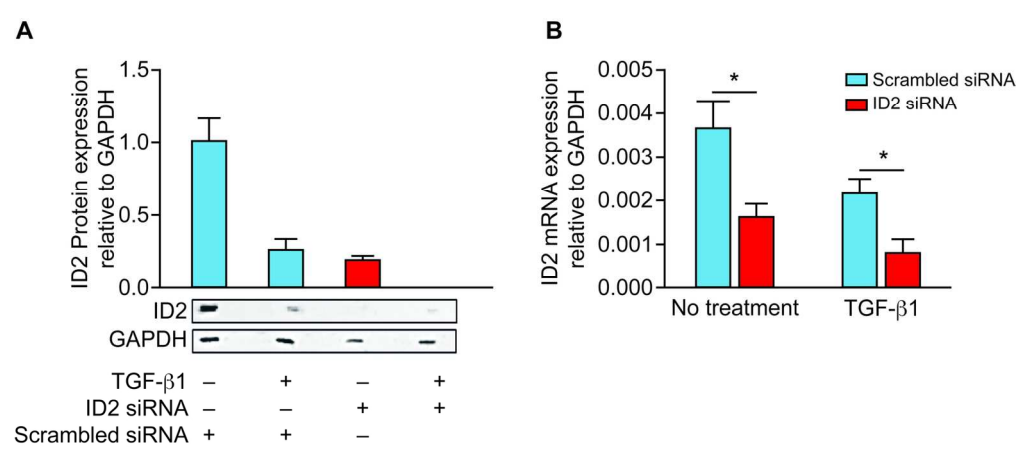
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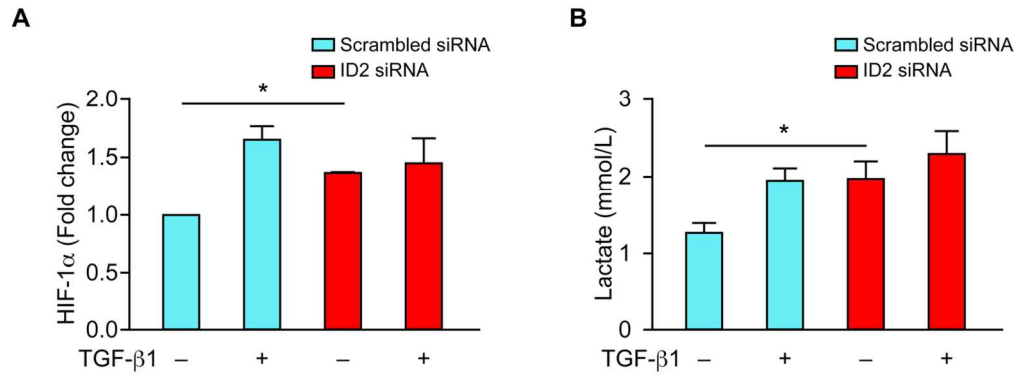
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