

1 **The depletion of MARVELD1 leads to placenta accreta via integrin**

2 **β 4-dependent trophoblast cell invasion**

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13 **KEY WORDS: MARVELD1; placenta accrete; integrin β 4; cell adhesion; trophoblast**
14 **cell; cell invasion**

15

16 **Abstract**

17

18 The mammalian placenta is a remarkable organ. It serves as the interface between
19 the mother and the fetus. Proper invasion of trophoblast cells into the maternal
20 decidua is required for a successful pregnancy. Previous studies have found that the
21 adhesion molecule integrin $\beta 4$ plays important roles during trophoblast cell invasion.
22 Here, we found that the overall birth rate of the MARVELD1 knockout mouse is much
23 lower than that of the wild-type mouse ($P < 0.001$). In E18.5 MARVELD1 knockout
24 mice, we observed an over-invasion of trophoblast cells, and indeed, the pregnant
25 mice had a partial placenta accreta phenotype. The HTR8/SVneo cell line was used as
26 an in vitro model to elucidate the underlying mechanisms of MARVELD1-mediated
27 trophoblast invasion. We detected a diminished expression of integrin $\beta 4$ upon the
28 downregulation of MARVELD1 and enhanced migration and invasive abilities of
29 trophoblast cells both in vivo and in vitro. The integrin $\beta 4$ rescue assay also
30 supported the results. In conclusion, this study found that MARVELD1 mediated the
31 invasion of trophoblast cells via regulating the expression of integrin $\beta 4$.

32

33

34 Introduction

35

36 The mammalian placenta is a remarkable organ. The robust growth of the fetus
37 is dependent on it. It forms the interface between the mother and fetus and is
38 needed for gas and nutrient exchange as well as waste disposal. In addition, the
39 placenta acts as a barrier against the maternal immune system by producing
40 hormones and growth factors(Rossant and Cross, 2001; Watson and Cross, 2005). In
41 humans and mice, the mature placenta is composed of three major layers: the outer
42 layer is called the maternal decidua, which contains decidual cells of the uterus as
43 well as the maternal vasculature that mediates blood exchange with the
44 implantation site. The middle layer acts as a “junctional region”, which is important
45 for the attachment of the placenta to the uterus; it contains trophoblast cells that
46 invade the maternal decidua. The inner layer is called the labyrinth, which is
47 composed of an abundance of branched villi for efficient gas and nutrient
48 exchange(Rossant and Cross, 2001). Although the detailed architecture of the
49 placenta in humans and mice are somewhat different, their overall structures and
50 the molecular mechanisms are quite similar(Rossant and Cross, 2001). Thus, the
51 mouse is an ideal model for studying placental development.

52 During placenta development in humans, the cytotrophoblast cells from anchor
53 villi differentiate into extravillous trophoblasts (EVTs), lose its proliferative phenotype,
54 and gain an invasive phenotype(Irving et al., 1995), forming three major subtypes:
55 villous cytotrophoblasts (CTBs), syncytiotrophoblasts and EVTs(Fitzgerald et al., 2008;
56 Gude et al., 2004; Lash, 2015). Similar to cancer progression, which involve cellular
57 movement into foreign tissue, trophoblast invasion into the maternal decidua is
58 tightly controlled by many adhesion molecules, including integrins(Damsky et al.,
59 1992; Damsky et al., 1994), matrix metalloproteinases (MMPs) and
60 metalloproteinases inhibitors (TIMPs) (Anacker et al., 2011; Bischof et al., 2000;
61 Godbole et al., 2011). In particular, cytotrophoblast cell invasiveness is associated

62 with different cell surface molecules, which include integrins. Integrins are a family
63 of heterodimeric transmembrane receptors that are formed by two different chains,
64 the α (alpha) and β (beta) subunits, and are expressed on the basal cell surface; it
65 mediates cell adhesion to extracellular matrix (ECM) through the amino acid
66 sequence Arginine-Glycine-Aspartic acid (RGD) (Ruoslahti, 1991; Ruoslahti, 1996).
67 Integrins play important roles in cell adhesion and migration. During the invasion
68 process, the expression of integrin $\alpha 1\beta 1$ and $\alpha 5\beta 1$ are upregulated whereas integrin
69 $\alpha 6\beta 4$ is downregulated (Damsky et al., 1994; Maltepe and Fisher, 2015).

70 Cellular invasion during placenta development, especially cytotrophoblast
71 differentiation, is a complex process that is tightly regulated. Proper invasion of
72 trophoblast cells into the maternal decidua is required for successful
73 pregnancy (Huppertz, 2008). Disruption of this tightly controlled process can lead to
74 placental deficiencies, resulting in pregnancy complications (Lash, 2015) such as early
75 miscarriage (Hustin et al., 1990), late miscarriage (Ball et al., 2006),
76 preeclampsia (Pijnenborg et al., 1991), fetal growth restriction (Khong et al., 1986),
77 preterm birth (Kim et al., 2003), and placenta accreta (Khong and Robertson, 1987).
78 Placenta accreta is a disorder of human placentation characterized by the abnormal
79 attachment or invasion of placental tissue into the underlying uterine musculature
80 (Dashraath and Lin, 2016; Jauniaux et al., 2016; Luke et al., 1966). Despite the
81 importance of cytotrophoblast cell invasion in placenta development, very little is
82 known about the factors that control this process in vivo.

83 Nuclear factor MARVELD1 (MARVEL domain-containing 1) is a novel tumor
84 suppressor candidate, which is widely expressed in human normal tissues but is
85 down regulated in multiple carcinomas by promoter methylation (Wang et al., 2009).
86 Previous studies have found that mouse MARVELD1 is a nuclear protein that inhibits
87 cell migration (Zeng et al., 2011). Notably, in non-small cell lung cancer (NSCLC),
88 MARVELD1 regulates the balance of integrin $\beta 1/\beta 4$, integrin $\beta 1/\beta 4$ -mediated cell
89 surface ultrastructure, and EMT (Yao et al., 2015). Here, we addressed the role of

90 MARVELD1 in mouse placenta development. We demonstrate that the knockout of
91 MARVELD1 in mouse placenta induced the downregulation of integrin β 4 and further
92 suppressed integrin β 4-mediated cell adhesion; therefore, this novel nuclear factor
93 plays an essential role in the regulation of integrin β 4 and associated functions.
94 These molecular mechanism triggers the over-invasion of trophoblast cells, which
95 leads to placenta accreta and a difficult birthing process in *MARVELD1*^{-/-} mice. This
96 study accelerated our efforts in understanding the critical role of the placenta in
97 successful pregnancies.
98

99 **Materials and methods**

100

101 **Cell lines and reagents**

102 Human cell lines NIH/3T3, NCI-H460, MDA-MB-231, NCI-H28, NCI-H2452,
103 HTR-8/SVneo, IRR-MRC-5, and WI38 were purchased from the American Type Culture
104 Collection (ATCC, Manassas, VA). Stable cell lines NIH/3T3-MARVELD1-V5 and
105 NIH/3T3-PC cells were maintained by our lab. Antibodies against MARVELD1
106 (ab91640), Cytokeratin18 (ab668), and integrin β 4 (ab182120) were purchased from
107 Abcam (Cambridge, MA). Antibodies against V5 (#13202) were purchased from Cell
108 Signaling Technology. Antibody against GAPDH (TA-08) was purchased from ZSGB-BIO
109 (ZSGB-BIO, Beijing, China). Laminin (L4544) was purchased from Sigma, TGF- β 1
110 (240-B) was purchased from R&D, Lipofectamine[®] 2000 transfection reagent
111 (Cat#11668019) and cell tracker (Cat#C34552) were purchased from Invitrogen,
112 TRizol reagent (Cat#11667165001) and FastStart Universal SYBR Green Master (ROX)
113 (Cat#04913850001) were purchased from Roche, the PrimeScript[™] RT reagent Kit
114 with gDNA Eraser (Cat#RR047Q) was purchased from Takara, and the Dual-Luciferase
115 reporter assay system (Catalog no. E1910) was purchased from Promega.

116

117 **MARVELD1 knockout mice**

118 All animal experiments were performed in strict accordance with the
119 recommendations in the Guide for the Care and Use of Laboratory Animals from the
120 Harbin Institute of Technology. The MARVELD1 knockout mice were generated by
121 Biocytogen (Beijing, CHINA) by injecting conditional MARVELD1 knockout murine ES
122 cells (conditional Cre-loxP target vector cassette) into blastocysts with a C57BL/6
123 background (C57BL/6J-Tyrc-2J) to obtain chimeric mice. MARVELD1 heterozygous
124 animals were backcrossed to wild-type C57BL/6 mice to obtain conditional
125 MARVELD1 knockout mice without the albino phenotype (Tyrc-2J). Then, the
126 conditional MARVELD1 knockout mice were crossed with Ella-Cre mice to obtain

127 MARVELD1 knockout mice. Mice were genotyped by PCR using primers that detected
128 wild-type and knockout sequences. Primers sequences are available in the Table S1.

129

130 **Cell culture and RNA interference**

131 HTR-8/SVneo cells were derived by transfecting the cells that grew from chorionic
132 villi explants of human first-trimester placenta with the gene encoding simian virus
133 40 large T antigen. These transfected trophoblasts were used for the study of
134 trophoblast biology and placental function. Cells were cultured following the
135 protocol from ATCC (Gibco, NY). Cells were cultured in RPMI-1640 medium with 5%
136 fetal bovine serum at 37 °C in an incubator with 5% CO₂.

137 For RNA interference, MARVELD1 siRNAs and scrambled siRNAs were designed by
138 the GenePharma Company. Cells were transfected with Lipofectamine® 2000
139 Transfection Reagent following the manufacturers' protocol. The knockdown
140 efficiency of the siRNAs was determined by real-time PCR.

141

142 **Reverse transcription and real-time PCR**

143 Total RNA was extracted and purified using the TRIzol reagent. The concentration of
144 each RNA sample was determined by using the NanoDrop 2000 spectrophotometer
145 (Thermo Fisher Scientific Inc.). One µg of total RNA was used as a template for
146 reverse transcription into cDNA using the PrimeScript™ RT reagent Kit with gDNA
147 Eraser according to the manufacturer's instructions. Primers are available in the
148 Table S1.

149 Quantitative real-time PCR was performed with FastStart Universal SYBR Green
150 Master (ROX) according to the manufacturer's instructions using the ViiTM 7
151 Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

152

153 **Western blotting**

154 Western blotting was performed as described previously. Briefly, total protein was

155 separated on a precast 12% polyacrylamide gel and blotted with antibodies for
156 integrin β 4 (diluted 1:1000), integrin β 1 (diluted 1:1000) and GAPDH (diluted
157 1:20000). Data were analyzed and quantified by using the Odyssey infrared imaging
158 system application software v3.0.

159

160 **Histological analysis**

161 For placental tissue, 5 μ m sections were used. Sections were collected and fixed in 4%
162 paraformaldehyde, dehydrated, embedded in paraffin and sectioned. Histological
163 sections were stained with hematoxylin and eosin (H&E) or used for other analyses
164 such as immunohistochemistry (IHC) and immunofluorescence (IF) staining assays.

165

166 **IHC and IF**

167 Sections were washed three times in PBST (PBS with 0.1% Triton X-100), blocked with
168 10% normal serum/PBST from the same species as the secondary antibody, and
169 incubated overnight at 4 °C with primary antibodies: Mouse anti-Cytokeratin
170 18(1:500), Rabbit anti-integrin β 4 (1:250), and MARVELD1 (1:100). The primary
171 antibodies were removed, and the sections were washed three times with PBST for 5
172 minutes each.

173 For IHC, 100-400 μ l of biotinylated secondary antibodies, diluted in TBST per the
174 manufacturer's recommendation, was added to each section. Then, the antibody
175 staining was developed using established procedures.

176 For IF, the antibodies were applied in the same manner as for cell staining. The
177 secondary antibodies were from Invitrogen. Stained sections were visualized using a
178 Zeiss AXIO Zoom.V16 Stereo Zoom Microscope.

179 For cell staining, cells were fixed in 4% paraformaldehyde for 15 minutes on ice,
180 blocked with 5% normal serum from the same species as the secondary antibody,
181 and stained using established procedures. Trophoblast cells were detected using anti
182 Cytokeratin18 (1:500) and anti-integrin β 4 (1:250) antibodies. Stained cells were

183 visualized using a Zeiss LSM 510 Meta Confocal Microscope.

184

185 **Transwell cell migration and invasion assays**

186 A total of 1.5×10^4 cells were seeded onto the upper chamber of 24-well Transwell
187 plates. Medium containing 10% FBS was placed in the lower chamber and served as a
188 chemoattractant. Cells were allowed to migrate for 18 hours at 37 °C, the cells on the
189 upper surface of the filter were removed by gently wiping with a cotton swab. The
190 cells that had migrated to the Transwell were fixed and stained with crystal violet.
191 The migrated cells were visualized by a microscope.

192 For the invasion assay, 1% Matrigel was added to the upper chamber before cell
193 migration, and cells were allowed to migrate for 24 hours at 37 °C.

194

195 **Cell adhesion assay**

196 A total of 5×10^3 cells labeled with cell tracker were plated onto 96-well plates coated
197 with laminin, and the cells were cultured in RPMI-1640 medium with 5% fetal bovine
198 serum at 37 °C in an incubator with 5% CO₂ for 3 hours.

199

200 **Chromatin immunoprecipitation (ChIP) assay**

201 A total of 1×10^7 NIH/3T3-MARVELD1-V5 cells were used for the ChIP assay. ChIP was
202 performed as described previously (Weinmann and Farnham, 2002). Promoter
203 regions were PCR amplified with specific pairs of primers that are listed in Table s1.

204

205 **Luciferase assay**

206 HTR-8/SVneo cells were cultured in 12-well plates (Corning Glass, Tewksbury, MA,
207 USA) and were transfected with 1 µg of PC or MARVELD1-V5 along with an integrin
208 β4 promoter construct. At 24 hours after transfection, luciferase activity was assayed
209 with 10 µl of lysate using the Dual-Luciferase reporter assay system from Promega
210 (Madison, WI, USA) and a Dynex (Sunnyvale, CA, USA) luminometer. The transfection
211 efficiency was normalized to Renilla luciferase activity. Luciferase activity was

212 measured using the CytoFluorplate 4000 Luminescence Microplate Reader (ABI,
213 Foster City, CA). Three transfection assays were performed to obtain statistically
214 significant data.

215

216 **Statistical analysis**

217 Statistical analyses were performed via two-tailed Student's t-test or one-way
218 ANOVA where indicated. $P < 0.05$ was considered as statistically significant, $P^* < 0.05$,
219 $P^{**} < 0.01$, and $P^{***} < 0.001$. Data from at least three independent experiments per
220 group were used for evaluation. Values are presented as the mean \pm SEM.

221

222 **Results**

223

224 **Generation of MARVELD1 knockout mice via the conditional Cre-loxP targeting**
225 **system**

226 In humans, the nuclear factor MARVELD1 is a potential tumor suppressor. Our
227 previous study found that MARVELD1 is a novel protein and is downregulated in
228 multiple cancers via methylation of its promoter. More importantly, it mediates cell
229 adhesion and migration by regulating integrin family molecules. To further
230 investigate the role of this novel protein in developmental processes, we generated
231 the MARVELD1 knockout mice using a conditional targeting vector (Fig. 1A). We
232 obtained *MARVELD1*^{+/+}, *MARVELD1*^{+/-} and *MARVELD1*^{-/-} mice (Fig. 1B). The
233 *MARVELD1*^{-/-} mice are able to develop into normal adults.

234

235 **MARVELD1 knockout mice have lower newborn offspring rate**

236 The knockout of MARVELD1 does not result in a distinct phenotype after birth. We
237 used *MARVELD1*^{-/-} mice to identify the phenotype of MARVELD1 deletion. At first, we
238 assessed MARVELD1 function by determining the birth rate of *MARVELD1*^{+/-}
239 intercrossed mice. The newborn offspring genotypes of 68 litters were counted.
240 Notably, 0.3749 ± 0.03082 were wild-type, 0.4746 ± 0.03068 were heterozygotes,
241 and 0.1505 ± 0.01998 were homozygotes. Compared with the wild-type offspring,
242 the birth rate of homozygotes offspring was significantly lower ($P < 0.001$) (Fig. 1C).
243 We also collected *MARVELD1*^{+/+} intercross, *MARVELD1*^{+/-} intercross, and *MARVELD1*^{-/-}
244 intercross newborn offspring genotypes to confirm the results. Compared with the
245 wild-type intercross group, the birth rate in the homozygous group was significantly
246 lower ($P < 0.01$) (Fig. 1D); this data indicated that the *MARVELD1*^{-/-} mice have lower
247 birth rates than that of wild-type mice. To further identify the reasons that impact
248 the knockout mice birth rate, we analyzed the E18.5 embryo numbers and the
249 newborn offspring pup numbers after intercrossing *MARVELD1*^{-/-} mice; compared
250 with the E18.5 embryo numbers, the newborn offspring pup numbers were

251 significantly lower ($P<0.001$) (Fig. 1E), which indicated an increased tendency to lose
252 pups before childbirth.

253

254 **The pregnant MARVELD1 knockout mice exhibited a placenta accreta phenotype**

255 In addition to decreased pup numbers in the MARVELD1 knockout mice after E18.5,
256 we also found that the placenta attached to the maternal uterus (Fig. 1F) and that
257 the newborn offspring exhibited long umbilical cords (Fig. 1G) by the time of birth.
258 These phenotypes suggested the MARVELD1 knockout mice, to a large extent,
259 exhibited placenta accreta. We further tested the expression levels of MARVELD1 in
260 the E18.5 wild-type mouse organs and found that MARVELD1 was highly expressed in
261 the placenta (Fig. 2A); additionally, the expression levels were increased in the
262 placentas from E10.5 to E18.5 (Fig. 2B), which is the time point that correlates with
263 the trophoblast cell invasion process. We further identified the localization of
264 MARVELD1 in wild-type E18.5 placentas. As shown in Fig. 2C, MARVELD1 was highly
265 expressed in the trophoblast cells, especially the trophoblast cells that tended to
266 migrate into the maternal decidua. These observations indicate that MARVELD1 plays
267 a role in trophoblast cell invasion.

268

269 **The trophoblast cells of the MARVELD1 knockout mouse placenta exhibited** 270 **increased cell invasion**

271 The high expression of MARVELD1 in the placenta and the trophoblast cells partially
272 revealed that MARVELD1 plays a role in trophoblast cell invasion. To further confirm
273 this hypothesis, we investigated trophoblast cell invasion in placenta accreta mice.
274 HE staining showed abnormal adhesion of the placenta to the myometrium (Fig. 3A).
275 Using immunohistochemical staining of Cytokeratin18, a trophoblast cell marker, we
276 observed that the maternal decidua was highly occupied by trophoblast cells in
277 placenta accreta mice (Fig. 3B). After we confirmed the placenta accreta phenotype,
278 the rate of placenta accreta was determined in *MARVELD1*^{-/-} female mice. In total,

279 45.95% of female *MARVELD1*^{-/-} mice exhibited the placenta accreta phenotype, and
280 70.59% of those mice were pregnant for the first time (Fig. 3C). To double confirm
281 the over-invasion of trophoblast cells in *MARVELD1* knockout placenta, the E18.5
282 placentas of wild-type mice and *MARVELD1* knockout mice were compared. We used
283 HE staining to examine the architecture of the junction layer, which contain the
284 trophoblast cells that invade the maternal decidua. The boundary of the junction
285 layer in wild-type mice was clear; in contrast, the boundary in *MARVELD1* knockout
286 mice was indistinct (Fig. 3D). Using immunohistochemical staining, we visualized a
287 clear difference of trophoblast cell invasion between E18.5 wild-type placenta and
288 *MARVELD1* knockout placenta. An increase in trophoblast cell invasion from the
289 E18.5 knockout placentas into the maternal decidua was observed (Fig. 3E). These
290 observations confirmed a strong connection between *MARVELD1* and the
291 over-invasion of trophoblast cells.

292

293 ***MARVELD1* was downregulated during trophoblast cell migration and invasion**

294 To further elucidate the contribution of *MARVELD1* during trophoblast cell invasion,
295 the HTR8/SVneo cell line was developed as an in vitro model. In human cell lines, the
296 *MARVELD1* expression level is higher in mesenchymal cell lines and is lower in
297 epithelial cell lines. The *MARVELD1* expression level in the HTR8/SVneo cells was in
298 the mid-range (Fig. 4A). Ten ng/μL TGF-β1 was added to HTR8/SVneo cells, and
299 afterwards, the invasion ability of the HTR-8/SVneo cells was improved (Fig. 4B).
300 Then, we tested the expression of epithelial markers, mesenchymal markers, integrin
301 β1, integrin β4, and *MARVELD1*. As shown in Fig. 4C, treatment with 10 ng/μL TGF-β1
302 led to a significant decrease in E-cadherin expression level ($P < 0.001$) and an increase
303 in the expression of mesenchymal markers N-cadherin and snail ($P < 0.001$). When the
304 invasion ability of HTR8/SVneo cells was improved, we detected a notable increase of
305 integrinβ1 and a significant decrease of integrin β4; *MARVELD1* was slightly but not
306 that significantly decreased. These results indicated that the HTR8/SVneo cell line

307 was an ideal model to assess MARVELD1-mediated trophoblast cell invasion.

308 We further used the Transwell chamber to select cells by their migration ability.
309 Cells above the membrane and below the membrane were collected separately.
310 Different cell numbers were used: 2×10^4 , 4×10^4 , 6×10^4 and 8×10^4 . MARVELD1
311 expression in the cells above the membrane and below the membrane was
312 significantly different at each cell density except 8×10^4 (Fig. 4D). Different time
313 points were also tested by plating 6×10^4 cells onto the chamber and collecting cells
314 above the membrane and below the membrane after 0, 18, 22, 26, and 36 hours.
315 The difference in MARVELD1 expression between the migrated cells and the
316 non-migrated cells exhibited the highest difference at 18 hours, and the difference in
317 expression diminished afterwards; there was no significant difference after 36 hours.
318 These data indicate that MARVELD1 is expressed at low levels in the migrated
319 trophoblast cells.

320

321 **MARVELD1 regulated trophoblast cell invasion via integrin $\beta 4$**

322 In human non-small cell lung cancer, we found that MARVELD1 mediated the balance
323 of integrin $\beta 1/\beta 4$ and the EMT phenotype (Yao et al., 2015). Since integrin $\beta 1/\beta 4$
324 belongs to integrin family, which participates in trophoblast cell invasion, we
325 transfected the HTR8/SVneo cells with siRNAs that can downregulate the mRNA
326 expression level of MARVELD1 to assess if MARVELD1 regulates the expression of
327 integrin $\beta 1/\beta 4$ in trophoblast cells. We speculated that MARVELD1 had the same
328 roles in trophoblast cells as in human non-small cell lung cancer. We found, however,
329 that the roles were not the same, but they were similar. Compared with the
330 scrambled siRNA-transfected HTR8/SVneo cells, MARVELD1 siRNA significantly
331 decreased the mRNA expression level of MARVELD1 ($P < 0.001$) as well as the
332 expression level of integrin $\beta 4$ ($P < 0.001$), but the expression level of integrin $\beta 1$ was
333 not significantly changed (Fig. 5A). The protein level of integrin $\beta 1/\beta 4$ correlated with
334 the mRNA results (Fig. 5B, 5C). These results indicated that MARVELD1 specifically

335 regulated the expression of integrin β 4 in trophoblast cells. To determine whether
336 this relationship between MARVELD1 and integrin β 4 was involved in the migration
337 and invasive ability of HTR8/SVneo cells, the cells were transfected with scrambled
338 siRNA, siRNA for MARVELD1, vector, siRNA for MARVELD1 and vector, integrin β 4,
339 and siRNAs for MARVELD1 and integrin β 4. The migration and invasive abilities were
340 positively correlated, and the abilities from highest to lowest were as follows:
341 siMARVELD1~siMARVELD1 +PC; scrambled siRNA~ scrambled siRNA +PC; integrin β 4
342 +siMARVELD1; and integrin β 4. Compared with the scrambled siRNA group, the
343 migration and invasive abilities were enhanced in the cells transfected with siRNA for
344 MARVELD1 (Fig. 5D). These results support the synergy between MARVELD1 and
345 integrin β 4 in the invasion of HTR8/SVneo cells.

346 Since integrin β 4 promoted its signaling events and mediated cell adhesion to
347 ECM, we also tested the adhesion ability of cells transfected with scrambled siRNA,
348 siRNA for MARVELD1, vector, siRNA for MARVELD1 and vector, integrin β 4, and
349 siRNAs for MARVELD1 and integrin β 4. The adhesion ability of each group was
350 negatively correlated with their migration and invasive abilities (Fig. 5E). These
351 results further support that MARVELD1 regulates trophoblast cell invasion via
352 integrin β 4-dependent cell adhesion.

353 As the invasion process of trophoblast cell is somewhat like an EMT process,
354 EMT markers were also tested to double confirm our hypothesis. As shown is Fig. 5F,
355 the E marker was down regulated when the invasive ability was increased, and there
356 were no significant changes in the M marker. These data fully confirm our previous
357 hypothesis.

358

359 **MARVELD1 increases the expression of integrin β 4 by enhancing its promoter** 360 **activity**

361 As the expression of integrin β 4 is correlated with MARVELD1, this raises the
362 question of how MARVELD1 regulates the expression of integrin β 4. We have

363 demonstrated before that MARVELD1 could specifically bind to the promoter of
364 integrin β 4 and activate it, which then enhanced the expression of integrin β 4 in
365 human cancer cell lines. To determine whether the same mechanism occurs in
366 mouse cells, NIH/3T3-MARVELD1-V5 cells were used in a ChIP assay. As shown in Fig.
367 6A, MARVELD1 specifically bound to the integrin β 4 promoter. Luciferase assays were
368 performed in HTR8/SVneo cells transfected with MARVELD1-V5 plasmids. As shown
369 in Fig. 6B, the cells transfected with MARVELD1-V5 resulted in significantly higher
370 integrin β 4 promoter activity than PC control cells. These data demonstrate that
371 MARVELD1 regulates the expression of integrin β 4 by enhancing its promoter
372 activity.

373

374 **Loss of MARVELD1 affects trophoblast cell invasion via integrin β 4 expression** 375 **changes in vivo**

376 The migration capability of HTR8/SVneo cells could be affected by the decreased
377 expression of MARVELD1 via its effects on the integrin β 4-mediated cell adhesion
378 pathway, thus explaining the over-invasiveness of trophoblast cells observed in
379 MARVELD1 knockout mice in vivo. The invasion of trophoblast cells is a precise
380 process that is regulated by complex molecules; therefore, we aimed to further
381 confirm the molecular mechanisms in vivo. In E18.5 placentas, the deletion of
382 MARVELD1 resulted in a decrease of the integrin β 4 (Fig. 6C) expression,
383 demonstrating an overall increase in its cell adhesion pathway. In Fig. 6D, we show in
384 E18.5 MARVELD1^{-/-} placentas that there was a large amount of trophoblast cells that
385 invaded into the maternal decidua compared to wild-type mice. The observed effect
386 of MARVELD1 knockout in the E18.5 placenta supports the notion that the deletion
387 of MARVELD1 downregulates the expression of integrin β 4 and promotes the
388 over-invasion of trophoblast cells (Fig. 7A).

389

390

391 **Discussion**

392

393 The function of MARVELD1, a novel nuclear factor that mediates cell adhesion and
394 migration in lung cancer, in mouse development is still unclear. Since gene knockout
395 mice are ideal models in which to study gene function, we developed the MARVELD1
396 knockout mouse to assess phenotypes after gene deletion, and more importantly, to
397 explore the molecular mechanisms of this novel protein. Our data indicated that the
398 knockout of MARVELD1 caused low birth rates but did not affect the numbers of
399 embryonic offspring. We identified a placenta accreta phenotype, characterized by
400 the attachment of the placenta to the maternal uterus, and the over-invasion of
401 trophoblast cells in MARVELD1 knockout mice. Consistent with these observations,
402 we found that the loss of MARVELD1 suppressed integrin β 4 expression and the
403 integrin β 4-dependent cell adhesion process, demonstrating that MARVELD1
404 mediates integrin β 4 expression and plays important roles in placenta development,
405 especially in trophoblast cell invasion (Fig. 7A, 7B).

406 MARVELD1 is a novel MARVEL domain-containing protein that is downregulated in
407 multiple human cancers(Wang et al., 2009). The MARVEL-domain containing proteins
408 such as MYADM(Aranda et al., 2011) and occludin(Huber et al., 2000) are involved in
409 cell adhesion and migration. Our previous studies have revealed that MARVELD1
410 regulates integrin β 1 through a pre-mRNA processing pathway(Wang et al., 2013) and
411 that MARVELD1 is involved in TGF- β 1-mediated EMT by regulating the balance of
412 integrin β 1 and β 4 expression in NSCLC cells(Yao et al., 2015). These findings indicate
413 that MARVELD1 plays a role in cell adhesion and migration in cancers. Since adhesion
414 and migration are basic cell characteristics, MARVELD1 is highly conserved between
415 different species, and therefore, we hypothesized that MARVELD1 plays a role during
416 mouse development.

417 To test the hypothesis, MARVELD1 knockout mice were developed. The
418 *MARVELD1*^{-/-} mice can develop into normal adults but have lower birth rates

419 compared to wild-type mice. During the birthing process in MARVELD1 knockout
420 mice, we visualized that the placentas were difficult to detach from the maternal
421 uterus due to the over-invasion of trophoblast cells. Cell migration is central to
422 multiple physiological processes, including embryonic development and cancer
423 metastasis. During both development and normal homeostasis, cells interact with
424 each other and the surrounding environment, including morphogen and the ECM, to
425 maintain tissue structure, organization and function(Paluch et al., 2016). During
426 embryonic development, cells migrate through interacting with their surrounding
427 environment; the tissue remodeling and morphogenesis processes involve the
428 migration of different cell types (Muthuswamy and Xue, 2012). The migration of cells
429 engaged in tissue-remodeling events contribute to cell invasion during development.
430 Rodents and human have similar hemochorial placenta, and trophoblast cell invasion
431 plays important roles in this type of placenta. The trophoblast cell invades into the
432 maternal uterus in search of spiral arterioles and veins. Trophoblast cells breach the
433 spiral arterioles and replace the endothelial and smooth muscle cells (Red-Horse et
434 al., 2006). These processes are important for both proper fetal perfusion and
435 attachment(Maltepe and Fisher, 2015). Previous studies have indicated that the
436 trophoblast cell invasion process is similar to epithelial-to-endothelial
437 transition(Maltepe and Fisher, 2015); E-cadherin and integrin $\alpha6\beta4$ are
438 downregulated during trophoblast cell invasion(Maltepe and Fisher, 2015).

439 Here, we identified that the downregulation of MARVELD1 decreases the
440 expression of integrin $\beta4$ and integrin $\beta4$ -dependent cell adhesion process both in
441 vivo and vitro, which correlates with the phenotype observed in MARVELD1
442 knockout mice. These in vivo and in vitro studies further elucidated the contribution
443 of MARVELD1 in placenta development. Notably, previously reported findings mainly
444 highlighted integrin $\beta4$ as an epithelial marker, and its expression was an indicator for
445 EMT. Based on our results, we suggest that integrin $\beta4$ is also an EMT initiator, and
446 this process is also under the control of MARVELD1 during placenta development.

447 Our study suggests a sequence of events that result in the over-invasion of
448 trophoblast cells; additional studies are required to further illustrate this process.
449 Although a lot has been discovered about the placenta in recent decades, there is a
450 lot more regarding placental abnormalities and pregnancy complications that remain
451 to be discovered. In summary, our study has elucidated a novel function of the
452 MARVELD1 gene in trophoblast cell invasion.
453
454

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461

462 **Author contributions**

463 YL and YC conceived the idea, designed experiments, provided the conceptual
464 framework for the study, and wrote the manuscript. HZ further contributed to
465 manuscript preparation. YC, HZ, FH, LY, and CQ performed the experiments. YC, HZ,
466 YZ and WL analyzed the data, PD contributed to conceptual evaluation of the project.

467

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573

574

576 **Figure legends**

577

578 **Figure 1. MARVELD1 knockout mice exhibit birthing difficulties.**

579 (A) Strategy for the modification of the MARVELD1 locus in ES cells and mice. Shown
580 in this figure is a schematic diagram of the targeting vector, the wild-type locus, and
581 the modified MARVELD1 locus before and after excision of the PGK-Neo cassette
582 with Cre recombinase.

583 (B) Genotype analysis of DNA from the tails of offspring animals for the modified
584 MARVELD1 allele. PCR reactions were carried out with primer pairs specific for the
585 wild-type MARVELD1 locus and the MARVELD1 knockout locus from which the neo
586 cassette had been excised by breeding with Cre mice (see text and Materials and
587 Methods for details). The sizes of the expected PCR products are indicated on the
588 right side, and the genotypes are indicated above each lane.

589 (C) Genotype distribution of progeny in *MARVELD1*^{+/-} intercross litters, n=68.

590 (D) Numbers of progeny in *MARVELD1*^{+/+} (n=21), *MARVELD1*^{+/-} (n=42), *MARVELD1*^{-/-}
591 (n=23) intercross litters.

592 (E) Numbers of progeny in *MARVELD1*^{-/-} intercross litters before birth (n=18) and
593 after birth (n=23).

594 (F) Difficult birth phenotype of the *MARVELD1*^{-/-} mother, and the successful
595 production of the wild-type mother.

596 (G) The long umbilical cord phenotype of newborn offspring from the *MARVELD1*^{-/-}
597 intercross.

598

599 **Figure 2. MARVELD1 is highly expressed in the placenta and invasive trophoblast**
600 **cells.**

601 (A) Relative expression of the mRNA level encoding MARVELD1 in E18.5 organs,
602 including brain, heart, liver, lung, kidney and placenta, was determined by real-time
603 RT-PCR. GAPDH mRNA was used to normalize the variability in template loading. The
604 data are reported as the mean ± SEM.

605 (B) Relative expression of mRNA encoding MARVELD1 in E10.5, E11.5, E12.5, E15.5
606 and E18.5 placentas.

607 (C) Immunohistochemical analysis of E18.5 placenta show that MARVELD1 is highly
608 expressed in the placenta and invasive trophoblast cells.

609

610 **Figure 3. *MARVELD1*^{-/-} placenta exhibits abnormal architecture and trophoblast cell**
611 **over-invasion**

612 (A) HE staining showing the attachment of the placenta to the maternal uterus from
613 a MARVELD1 KO dam.

614 (B) Immunohistochemistry of placentas from MARVELD1 KO dams for Cytokeratin18,
615 a trophoblast cell marker.

616 (C) The occurrence the dystocia phenotype in *MARVELD1*^{-/-} female mice, n=37; the
617 relationships between the dystocia phenotype and the times of pregnancy in
618 *MARVELD1*^{-/-} female mice, n=17.

619 (D) HE staining of the placenta from E18.5 MARVELD1 KO and control dams showing
620 the abnormal architecture of the junctional region in *MARVELD1*^{-/-} placenta.

621 (E) Immunohistochemistry of the placenta from E18.5 *MARVELD1*^{-/-} and control dams
622 for Cytokeratin18. Note the over-invasion of trophoblast cells in the *MARVELD1*^{-/-}
623 placenta.

624

625 **Figure 4. MARVELD1 and integrin β 4 are downregulated in HTR8/SVneo cells during**
626 **EMT**

627 (A) Relative expression of MARVELD1 mRNA in human cell lines was determined by
628 real-time RT-PCR. GAPDH mRNA was used to normalize the variability in template
629 loading. The data are reported as the mean \pm SEM.

630 (B) For the Transwell assay, 10 ng/ μ L TGF- β 1 was added to the cells, and the images
631 were acquired after 24 hours.

632 (C) The mRNA levels of epithelial markers, mesenchymal markers, integrin β 1,

633 integrin β 4, and MARVELD1 with/without TGF- β 1 determined by real-time RT-PCR.
634 (D) The mRNA level of MARVELD1 during cell migration stratified by cell number and
635 migration time. Non-migrated cells above the chamber and migrated cells below the
636 chamber were collected separately.

637

638 **Figure 5. MARVELD1 regulates trophoblast cell invasion via the integrin**
639 **β 4-mediated cell adhesion pathway**

640 siRNA for MARVELD1 was added to cells to downregulate MARVELD1 mRNA
641 expression levels.

642 (A) Relative expression of integrin β 1, integrin β 4, and MARVELD1 mRNA in cells was
643 determined by real-time RT-PCR. GAPDH mRNA was used to normalize the variability
644 in template loading.

645 (B) Western blot analysis of the expression of integrin β 1 and integrin β 4. GAPDH
646 was used as a loading control.

647 (C) Images of cells immunostained with antibodies against integrin β 4.

648 (D) Cells were labeled with cell tracker, and cell adhesion ability was identified after 3
649 hours.

650 (E) Transwell assay to analyze cell migration and invasion.

651 (F) Relative expression of the mRNA level encoding MARVELD1, integrin β 4,
652 E-cadherin and N-cadherin in cells. As determined by real-time RT-PCR, GAPDH
653 mRNA was used to normalize the variability in template loading.

654

655 **Figure 6. MARVELD1 increases the expression of integrin β 4 by enhancing its**
656 **promoter activity**

657 (A) A ChIP assay was performed by using HTR8/SVneo cell lysate. Chromatin was
658 immunoprecipitated using a V5-tag specific antibody. PCR was carried out using
659 specific primers for the integrin β 4 promoter.

660 (B) Cells were transfected with the integrin β 4 (-1,594~-449) or the integrin β 4

661 (-1,594~-1,418) luciferase reporter plasmid. The firefly luciferase activity was
662 measured 24 hours post-transfection and normalized to Renilla luciferase activity.
663 Values represent the mean \pm SEM of three independent experiments.

664 (C) Embryonic side: images of E18.5 *MARVELD1*^{+/+} and *MARVELD1*^{-/-} placenta sections
665 immunostained with antibodies against integrin β 4 (green) and Cytokeratin18 (red)
666 as well as DAPI staining (blue).

667 (D) Maternal side: images of E18.5 *MARVELD1*^{+/+} and *MARVELD1*^{-/-} placenta sections
668 immunostained with antibodies against integrin β 4 (green) and Cytokeratin18 (red)
669 as well as DAPI staining (blue).

670

671 **Figure 7. Proposed models for MARVELD1-mediated trophoblast cell invasion in**
672 **vitro and in vivo**

673 (A) Working model of MARVELD1 in mediating in vivo trophoblast cell invasion.
674 When MARVELD1 is knocked out, the expression level of integrin β 4 is
675 downregulated and the adhesive ability of cells is suppressed, which boost cell
676 migration and invasion, leading to trophoblast cell over-invasion and the placenta
677 accreta phenotype in *MARVELD1*^{-/-} mice.

678 (B) Underlying molecular mechanisms. MARVELD1 binds to the integrin β 4 promoter
679 to activate its transcription. Then, integrin β 4 upregulates the signaling processes
680 that facilitate cell adhesion and suppress cell migration and invasion.

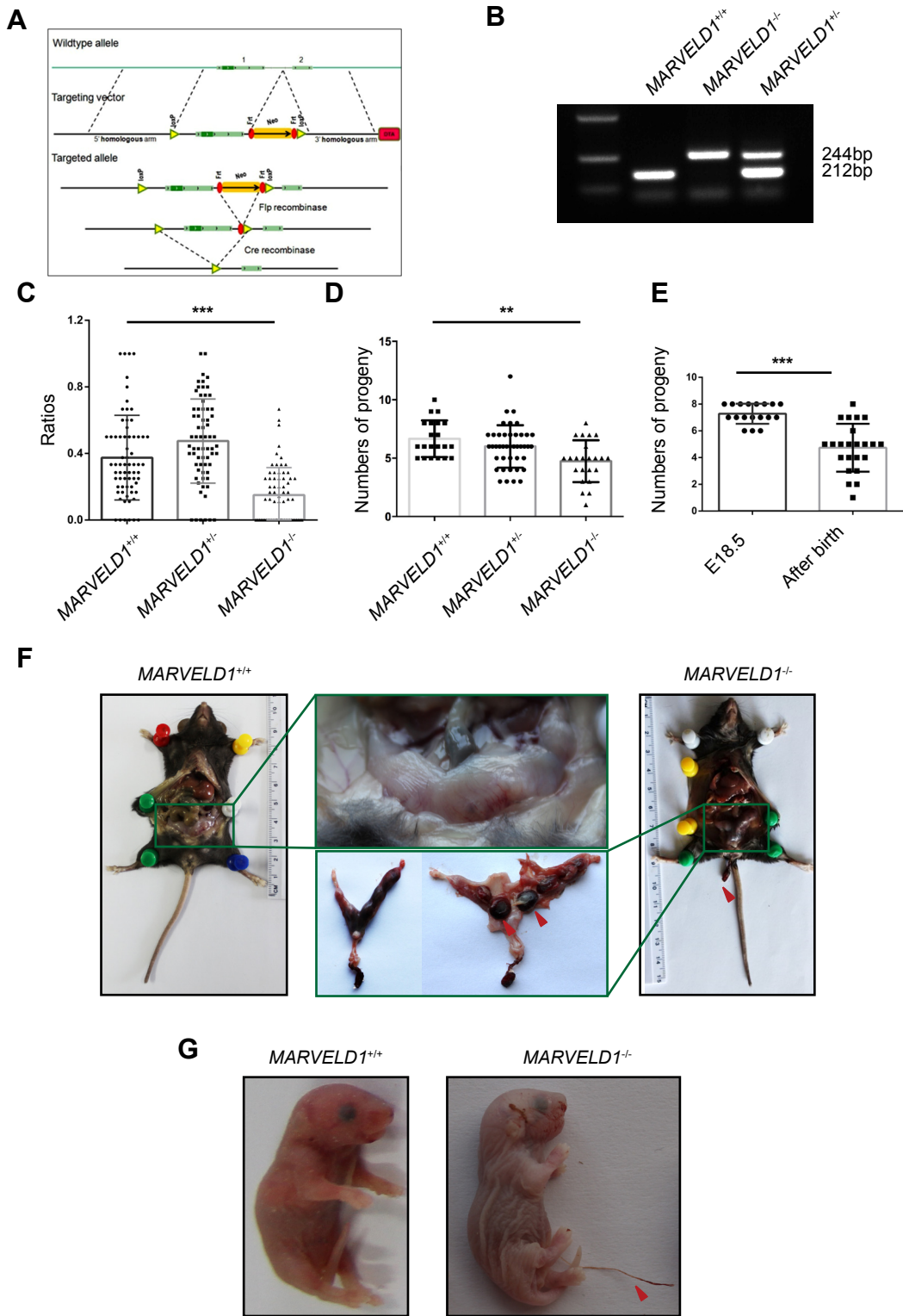


Figure 1

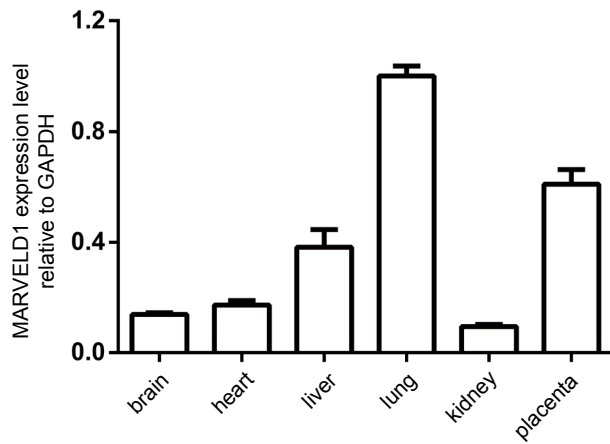
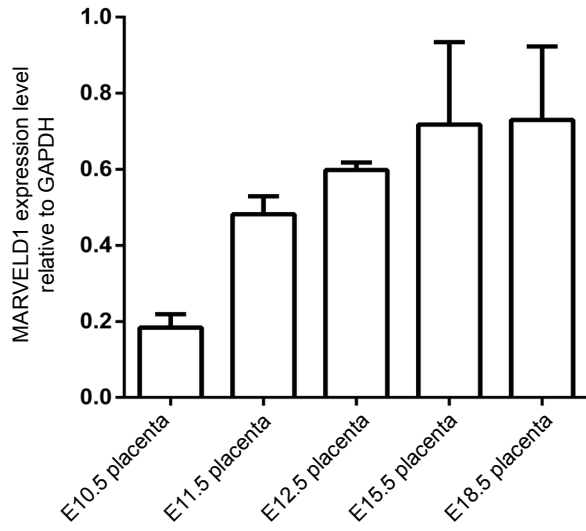
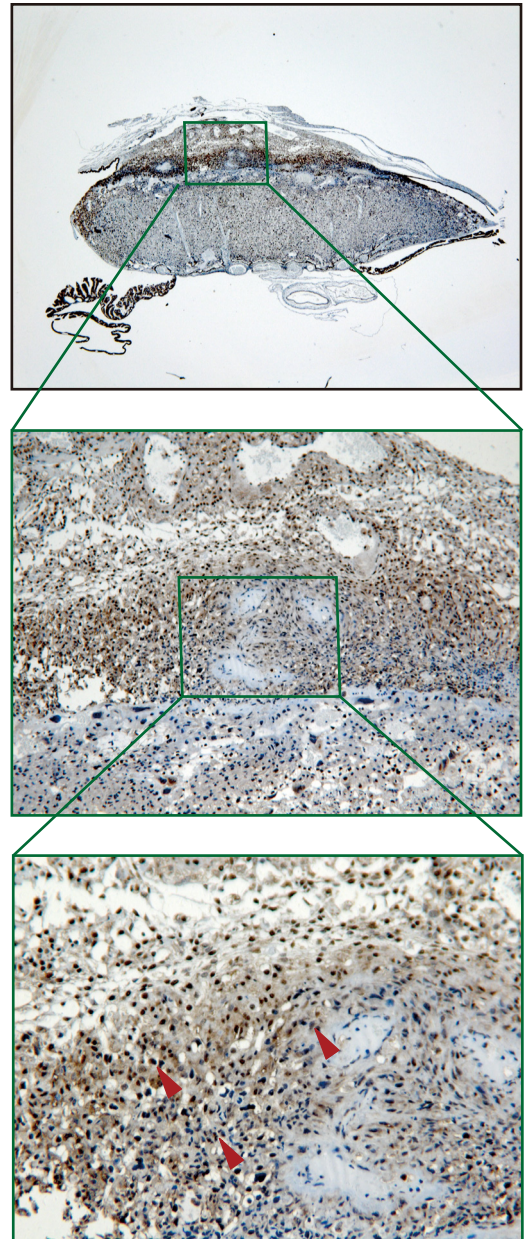
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Figure 2

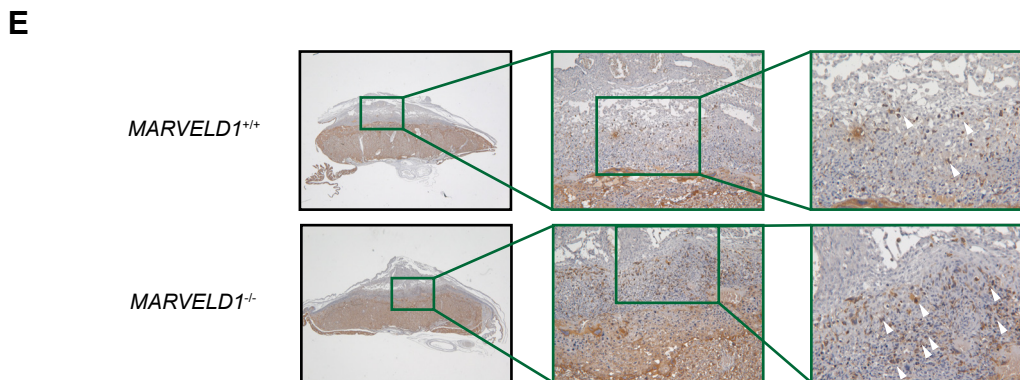
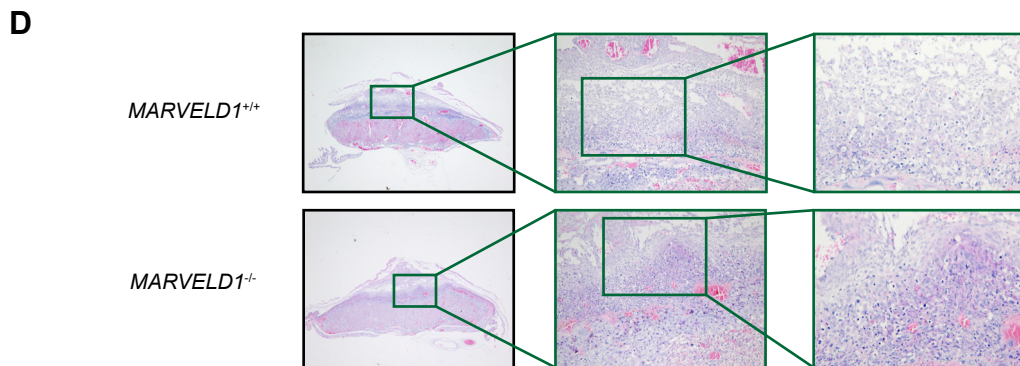
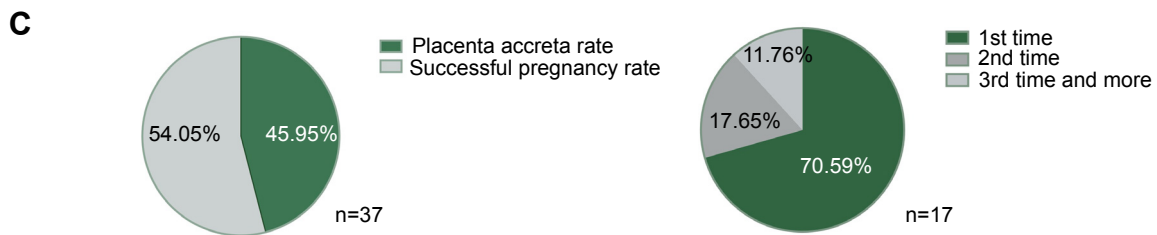
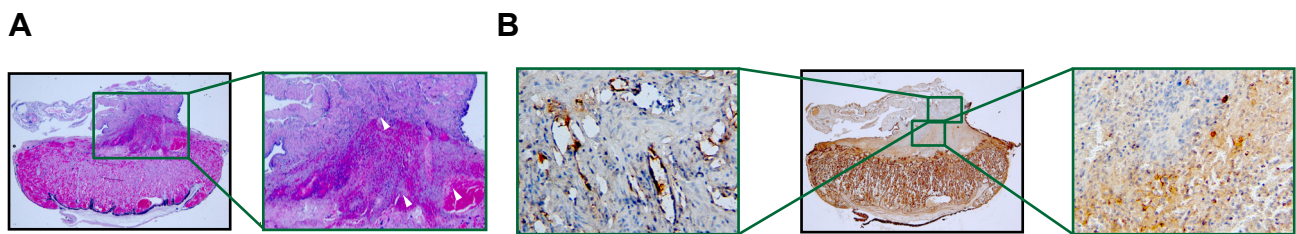
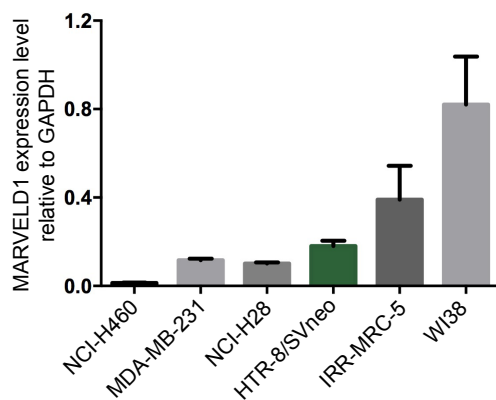
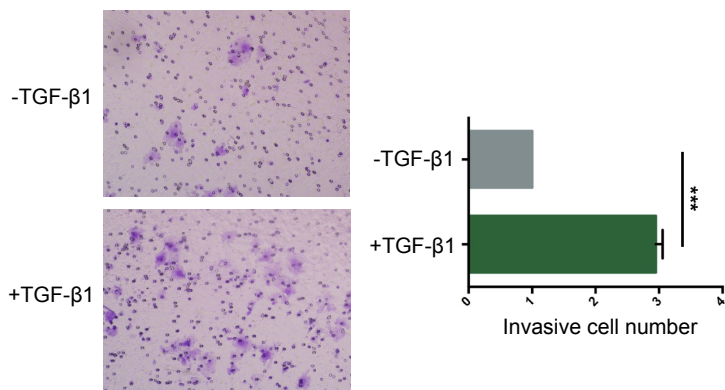
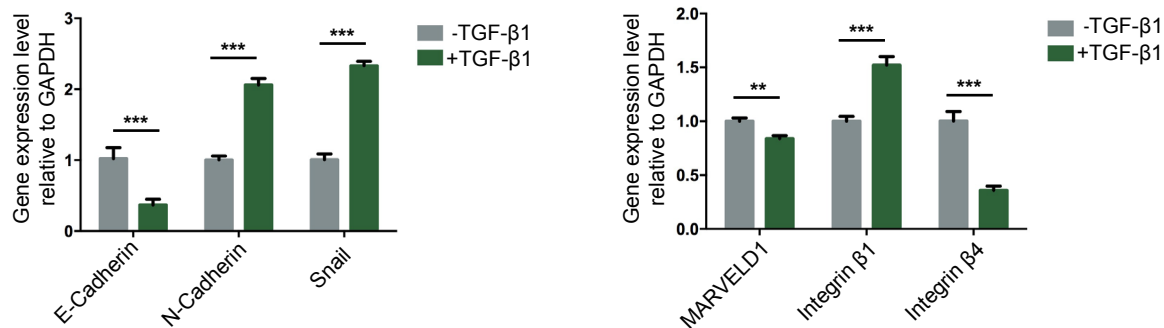
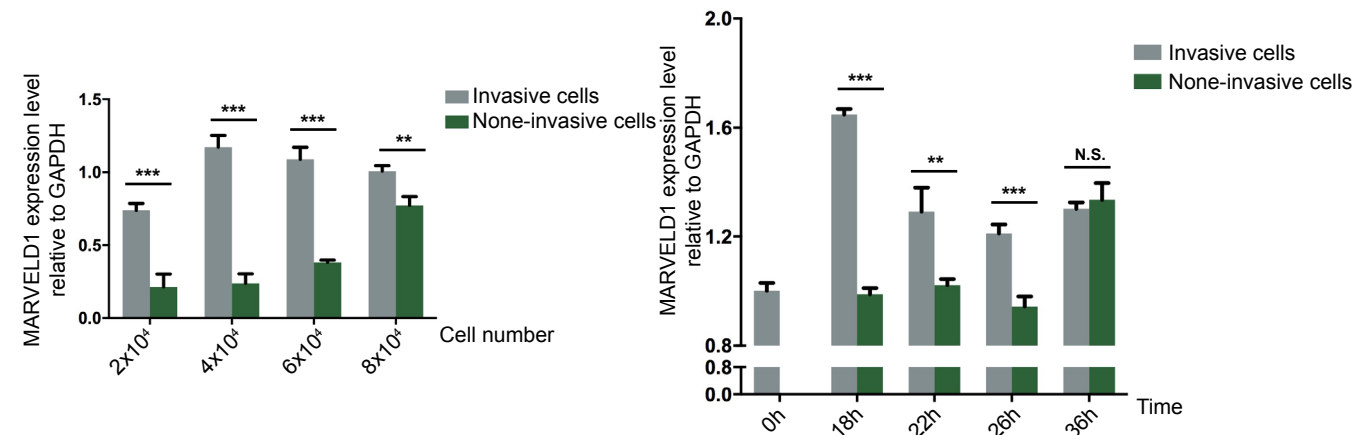


Figure 3

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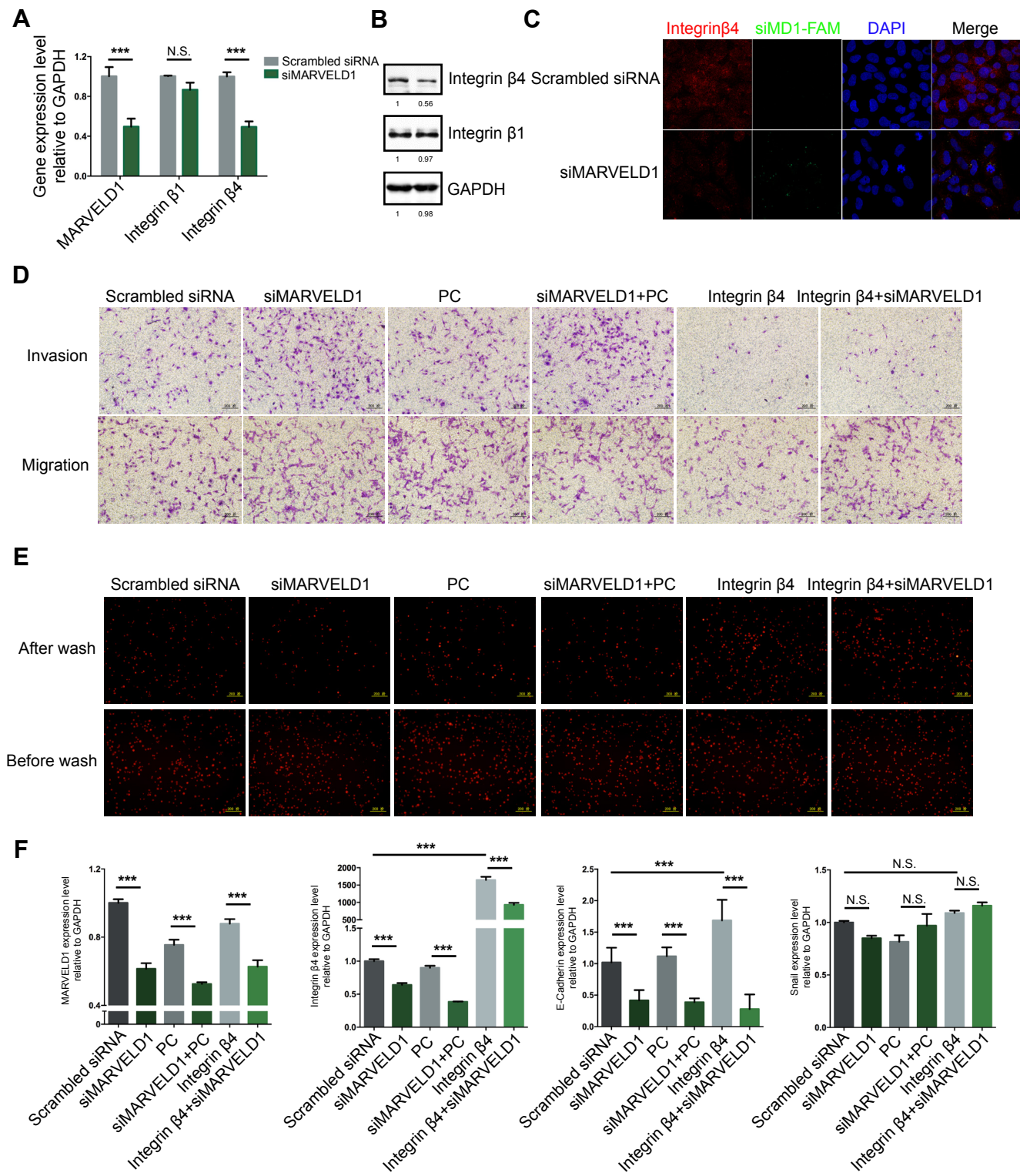


Figure 5

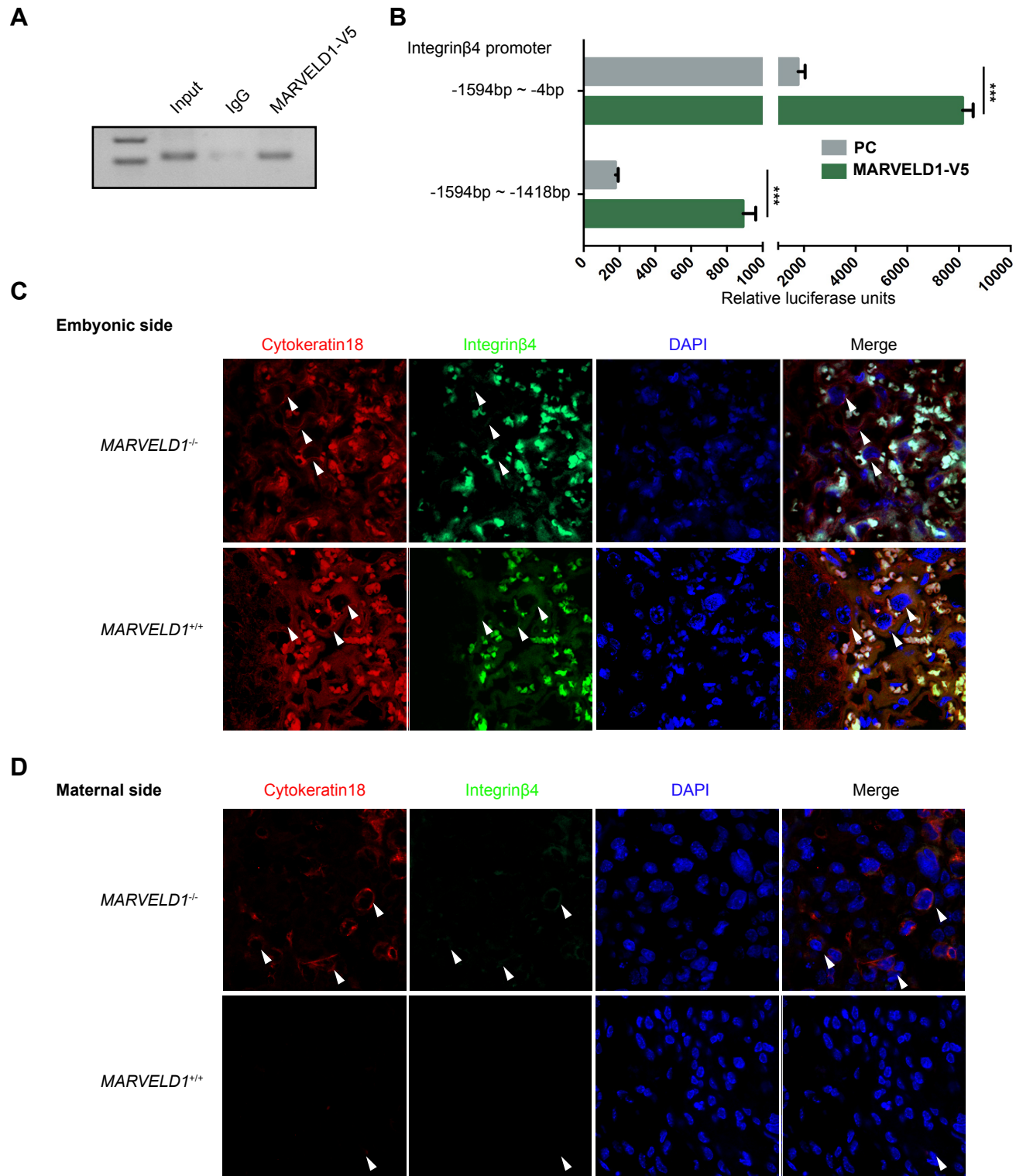


Figure 6

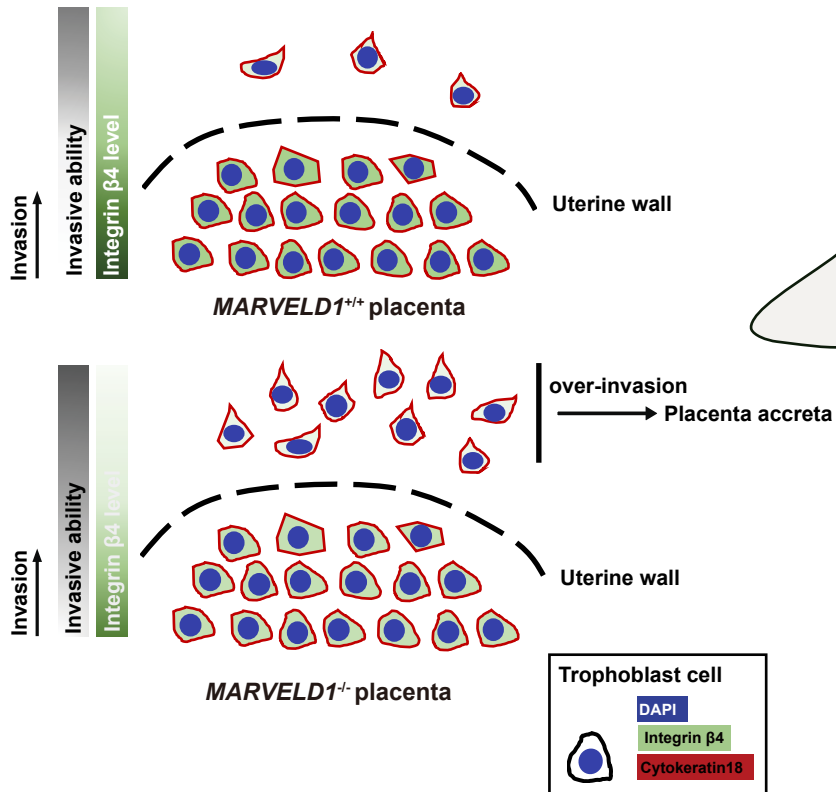
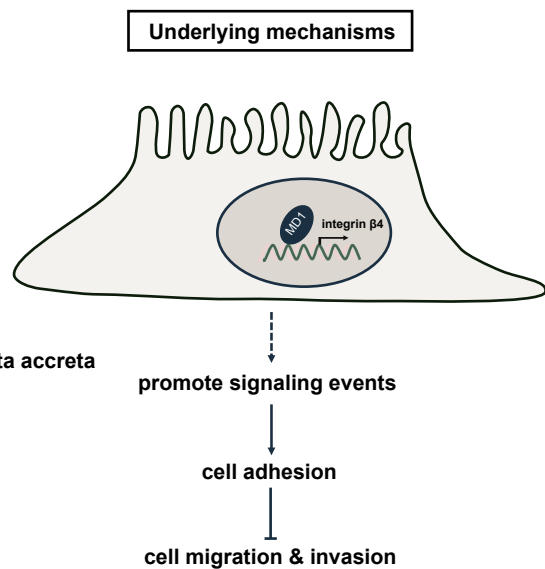
A**B**

Figure 7