# Genetically edited human placental organoids cast new light on the role of ACE2 in placental development

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## Abstract

ACE2 expression is altered in pregnancy disorders and *ACE2* gene variants are associated with several major pregnancy complications including small-for-gestational-age, fetal growth restriction and preeclampsia. This study utilised gene-editing to generate both *ACE2* knockout and *ACE2* rs2074192 placental organoids, facilitating mechanistic studies into the role of *ACE2* in placental development, and the effect of fetal carriage of *ACE2* rs2074192 CC, CT and TT genotypes. Parameters of cell and organoid growth were measured, together with qPCR, Western Blotting, and ELISA assessments, in all groups from both organoid models. Here, we report that *ACE2* knockout results in delayed placental cell growth and increased cell death. *ACE2* knockout organoids had lower ACE protein expression, reduced organoid diameters and asymmetrical growth. Placental organoids with the ACE2 rs2074192 TT genotype had significantly higher expression of *ACE2* mRNA and ACE2 protein with elevated ACE2:ACE expression ratio and no change in ACE protein expression. Despite increased expression of ACE2 protein, ACE2 enzyme activity was significantly decreased in ACE2 rs2074192 TT placental organoids. TT organoids also had reduced diameters and asymmetrical growth. Our research provides new molecular understanding of the role of ACE2 in placental development, with potential implications for pregnancy in carriage of the *ACE2* rs2074192 gene variant.



## Key words

ACE2, SNP, placenta, pregnancy, organoid, CRISPR, angiotensin converting enzyme 2, ACE, trophoblast

47 # Introduction

48 Interest in previously little-known angiotensin-converting enzyme 2 (ACE2) has escalated with
49 the COVID-19 pandemic and the knowledge that ACE2 is the SARS-CoV-2 receptor. However,
50 the role of ACE2 in the placenta remains understudied, despite its evident importance in
51 pregnancy. Pregnant women have higher circulating levels of ACE2 compared to non-pregnant
52 women[1]. ACE2 abundance is altered in pregnancy disorders, including preeclampsia, small for
53 gestational age (SGA) and fetal growth restriction[1, 2, 3, 4, 5, 6, 7], and *ACE2* gene variants are
54 associated with several major pregnancy
55 complications[1, 5, 8]. Genetic factors account for two
56 thirds of the phenotypic variation in circulating
57 ACE2[9]; specifically, *ACE2* single nucleotide
58 polymorphisms (SNPs) and haplotypes are
59 associated with altered abundance of Angiotensin II
60 (Ang II) and Angiotensin 1-7 (Ang 1-7), which are
61 the peptides produced by angiotensin converting
62 enzyme (ACE) and ACE2 catalytic activity,
63 respectively[10] (**Figure 1**).

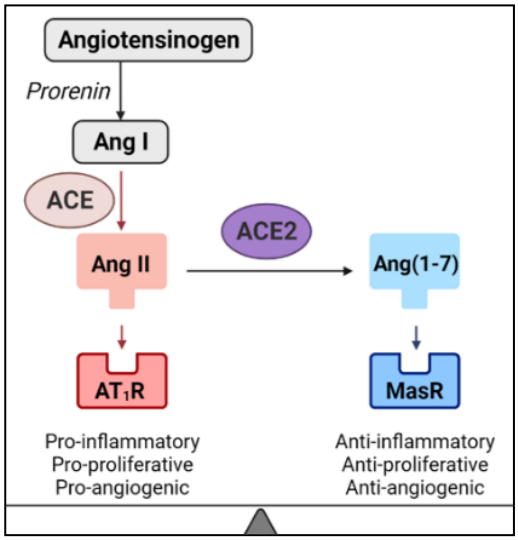


***Figure 1.*** *The ACE- and ACE2-mediated opposing arms of the renin-angiotensin system.*

64

65 The circulating renin-angiotensin system is a critical regulator of blood volume and systemic
66 vascular resistance. However, in addition to the circulatory system, local tissue-specific renin-
67 angiotensin systems exist, including within the placenta. The pro-inflammatory, pro-
68 proliferative arm of the placental renin-angiotensin system (shown in red in **Figure 1**),
69 mediated by ACE, promotes cell and tissue growth. This is opposed by an anti-inflammatory,
70 anti-proliferative arm (shown in blue in **Figure 1**), mediated by ACE2[3]. ACE and ACE2
71 maintain a finely tuned balance within the renin-angiotensin system to optimise placental

72 growth and function throughout healthy pregnancy. However, the expression and activity of
73 ACE and ACE2 enzymes, both systemically[1, 2] and in the placenta[3, 4, 5, 6, 7], is disturbed in many
74 pregnancy complications associated with renin-angiotensin system dysfunction.
75
76 Adequate ACE2 expression and activity, in both the mother and placenta, is necessary for
77 healthy pregnancy. In *ACE2*$^{-/-}$(*ACE2* knockout) mouse models, *ACE2* knockout dams have
78 higher systolic blood pressure[2, 4], decreased plasma Ang 1-7[2], and placentae have increased
79 levels of[2], and sensitivity to[4], Ang II. This results in alterations to the balance of ACE and ACE2
80 activity in the renin-angiotensin system. Furthermore, pups from *ACE2* knockout dams have
81 reduced weight[2, 4] and length[2], with lower pup to placenta weight ratios, indicating
82 compromised placental function. Clearly ACE2 is essential for healthy pregnancy but
83 dysregulation of ACE2 levels and activity, as well as ACE:ACE2 balance, is pathogenic[4, 11].
84 However, whilst murine research is informative, an *ACE2* knockout model of human placenta
85 has never been established.
86
87 As previously mentioned, *ACE2* gene variants (including single nucleotide polymorphisms
88 [SNPs]) alter expression of renin-angiotensin system components. A SNP is a single base
89 substitution at a specific chromosome location. Many SNPs in the *ACE2* gene have been
90 associated with disease states[5, 8, 12, 13, 14]. In particular, *ACE2* rs2074192 is associated with
91 increased incidence of diseases characterised by renin-angiotensin system dysregulation[12, 13,
92 14, 15], including an increased susceptibility to COVID-19 and increased disease severity[13, 14].
93 Importantly for pregnancy, SGA, a condition where neonatal birthweight is lower than
94 expected for their gestational age, has been associated with *ACE2* rs2074192 (C to T
95 polymorphism) which, when carried by the fetus, increases the risk of SGA by 23-fold[5]. The
96 placenta, a product of conception, is genetically identical to the fetus. As such, a fetal SNP will

97 also be present in the placenta. Despite strong associations between *ACE2* rs2074192 frequency
98 and disease state, and the estimated frequency of at least one copy of this SNP in one third of
99 the global population[16], research is mostly limited to association studies but not its mechanisms
100 of action.
101
102 In this study, we utilised gene-editing to generate *ACE2* knockout placental organoids and
103 *ACE2* rs2074192 placental organoids, creating the first induced SNP gene-edited organoids in
104 human placenta. We investigated the role of *ACE2* in placental development by profiling
105 expression of *ACE2* mRNA and protein, ACE protein, ACE2 enzyme activity and parameters
106 of cell and organoid growth in both placental organoid models. Elucidating the role of *ACE2*
107 in the placenta will allow inferences to be made regarding its overall contribution to pregnancy
108 health, as well as its possible role in other physiological and pathological states that involve
109 the renin-angiotensin system.
110
111

# Methods

Trophoblast stem cells (TSC) were isolated from n=3 patients and grown to confluence. At this point, TSCs from each patient were separated into two groups:

1. *ACE2*$^{-/-}$ (*ACE2* knockout – hereafter referred to as *ACE2*$^{+/+}$, *ACE2*$^{+/-}$ or *ACE2* KO)
2. *ACE2* SNP (rs2074192 – hereafter referred to by genotype, i.e. CC, CT or TT)

The generation of gene-edited organoids from each patient was replicated three times, thus a final n=9 independently generated organoids for each genotype group (see **Figure 2**).

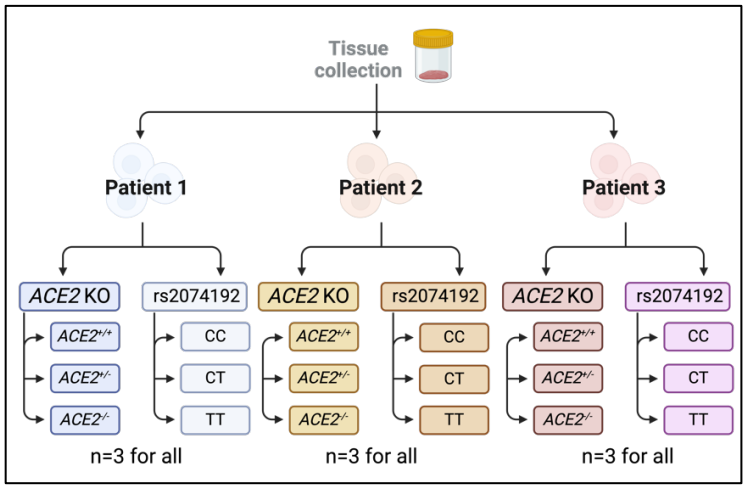


***Figure 2**. Method describing gene-editing to generate different TSC groups.*

As *ACE2* is on the X chromosome, only placentae from female fetuses were included to allow analysis of a heterozygous group (*ACE2*$^{+/-}$).

## Tissue collection

First trimester (6-7 weeks' gestation) human placentae were obtained with informed consent from women undergoing elective termination of pregnancy at the Pregnancy Advisory Centre in the Queen Elizabeth Hospital, in Woodville, South Australia. Ethics approval was obtained

129 from the Central Adelaide Local Health Network Human Research Ethics Committee,
130 HREC/16/TQEH/33, Q20160305. Placentae were collected within minutes of termination
131 upon which villous tissue was washed with Hanks' Balanced Salt Solution (HBSS; Gibco,
132 Sigma-Aldrich) and transported to the laboratory on ice.

133

134 ## Genotyping for fetal sex

135 Placentae from n=5 patients were genotyped for fetal sex high resolution melt curve analysis
136 of the gene that encodes amelogenin. Amelogenin is found on both the X (AMELX) and Y
137 (AMELY) chromosomes, the X allele features a 3 bp deletion in exon 3 allowing the
138 identification of samples with only X chromosomes (female) or X and Y chromosomes (male).
139 Forward primer 5′-CCCTGGGCTCTGTAAAGAATAGTG-3′, reverse primer 5′-
140 ATCAGAGCTTAAACTGGGAAGCTG-3. qPCR was performed using SSOFast EvaGreen
141 Supermix (Bio-Rad, CA), primers at 250 nM final concentration, 5 ng of DNA per reaction, on
142 a Bio-Rad CFX384 Real-Time PCR System. Cycling conditions: initial denaturation 98°C 30
143 s, 40 cycles of 98°C for 5 s and 60°C for 5 s. High resolution melt curve analysis was performed
144 from 65°C to 85°C with a 0.2°C increment every 10 s. Melt curve between 65°C and 70°C was
145 analysed using Bio-Rad Precision melt software (Bio-Rad, CA) to identify sex genotypes. N=3
146 of these placentae, determined to be from female fetuses, were selected for use in this study.

147

148 ## Isolation and cultivation of TSCs

149 TSCs were isolated from first trimester placentae according to Dietrich, *et al.*[17] Briefly, first
150 trimester villous tissue was washed in HBSS (4°C) prior to manually isolating villus structures
151 for further processing. Villi in HBSS were centrifuged (1000 rpm, 1 min) prior to three
152 consecutive enzymatic digest steps and density gradient centrifugation as per Haider, *et al.*[18].

153 After careful collection of cells between the 35% and 50% Percoll layers, cells were thoroughly
154 washed with HBSS before seeding in fibronectin-coated culture plates using a culture medium
155 to promote stemness. As per Dietrich, *et al.*[17], TSC medium contained DMEM/F12 (Gibco)
156 supplemented with 1×B-27 (Gibco), 1×Insulin-Transferrin-Selenium-Ethanolamine (ITS-X;
157 Gibco), 1 µM A83-01 (Tocris), 50 ng/ml recombinant human epidermal growth factor (rhEGF;
158 R&D Systems), 2 µM CHIR99021 (Tocris) and 5 µM Y27632 (Santa Cruz).

159

160 ## Generation of plasmids

161 To generate modified ACE2 organoid models, two distinct gene editing strategies were
162 employed. Firstly, a modified pDG459 plasmid (Addgene #100901) was designed to induce a
163 complete *ACE2* knockout (KO). This entailed the precise targeting of regions approximately
164 100 base pairs upstream and downstream of the first and last *ACE2* exons, resulting in the
165 excision of promoter, regulatory, and coding sequences. Secondly, a modified PEA1-Puro
166 plasmid (Addgene #171991) was designed to introduce an intronic *ACE2* single nucleotide
167 polymorphism (SNP), specifically rs2074192. The SNP was targeted using the PE3 strategy,
168 which incorporated a downstream nicking single-guide RNA (sgRNA) positioned 72 base pairs
169 from the primary cleavage site, adhering to the recommended range of 40-90 nucleotides[19].
170 Construct assembly was achieved through *BbsI*-mediated golden gate assembly of
171 phosphorylated and annealed oligonucleotide guide sequences (Thermo Fisher), following
172 optimised protocols for PEA1 or pDG459 assembly[20, 21]. Subsequent plasmid purification was
173 performed using the QIAprep Spin Miniprep Kit (Qiagen), and validation of oligonucleotide
174 insertion was carried out through Sanger sequencing (AGRF). Oligonucleotide sequences are
175 listed in **Table 1**.

176

177 ***Table 1.*** *Sequences of oligonucleotides used to generate plasmids for organoid models.*

| **Oligonucleotide** | **Oligonucleotide Sequence 5'-3'** |
|---|---|
| ACE2 KO Guide Promoter | caccgCTGTCATTTCAGAATAATGCT |
| ACE2 KO Guide Promoter | aaacAGCATTATTCTGAAATGACAGc |
| ACE2 KO Guide Terminator | accgCCTGACAGCTCATCATGAGAgt |
| ACE2 KO Guide Terminator | tgaaacTCTCATGATGAGCTGTCAGG |
| ACE2 SNP PE Nick sgRNA | accgGTAGATGTACTCTGACAGAAgt |
| ACE2 SNP PE Nick sgRNA | tgaaacTTCTGTCAGAGTACATCTAC |
| ACE2 SNP PE Spacer Guide | caccTGTGGAAATGTATAAATGGT |
| ACE2 SNP PE Spacer Guide | aaacACCATTTATACATTTCCACA |
| ACE2 SNP PE Repair | gtgcCAAATGAATAAATaCCAACCATTTATACATTTCCA |
| ACE2 SNP PE Repair | aaaaTGGAAATGTATAAATGGTTGGtATTTATTCATTTG |

178

179 ## TSC genetic modification

180 TSCs were transfected with *ACE2* KO and *ACE2* SNP plasmids using DNAfectin Plus
181 Transfection Reagent (abm), according to manufacturer's instructions. Briefly, media was
182 aspirated from the TSC culture wells prior to the addition of the DNA-transfection complex.
183 Control wells received only the transfection reagent without DNA plasmid. After 6 h the
184 transfection solution was removed, and complete medium was added. After 24 hours,
185 puromycin (0.2 µg/mL) was added to culture media to begin antibiotic selection of successfully
186 transfected cells. Puromycin selection continued for 14 days of culture (until cells reached
187 confluence).

188

189 ## Organoid formation

190 Placental organoids were established as per Haider, *et al.*[18], with minor modifications. Briefly,
191 gene-edited TSCs were trypsinised (TrypLE, Gibco) and washed in ice-cold advanced

192 Dulbecco's Modified Eagle Medium (DMEM, Gibco). Cells were resuspended in organoid
193 media: ice-cold advanced DMEM supplemented with 1×B-27, 1×ITS-X, 10 mM HEPES
194 (Gibco), 2 mM glutamine (Gibco), 1 µM A8391, 100 ng/ml rhEGF and 3 µM CHIR99021.
195 Vitrogel (ORGANOID-1, The Well BioScience) was added to reach a 2:1 Vitrogel to organoid
196 media ratio. 75µL of the resultant mixture was plated in a 6-well culture plate, forming a dome
197 in the centre of the well. After incubation (37°C, 2 h), 1.5mL organoid media (37°C) was added
198 to each well. Media was changed every 5 days until organoids reached confluence, at which
199 point they were harvested for RNA and protein experiments.

200

201 ## Genomic DNA (gDNA) extraction

202 gDNA extraction protocol was adapted from Ghatak, *et al.*[22]. Prior to organoid generation, a
203 subset of TSCs from each gene-edited patient sample was harvested and gDNA was extracted
204 by incubating cells with a phenol:chloroform:isoamyl alcohol solution (25:24:1). After
205 centrifugation (10 mins, 10000 xg, 4°C), the upper aqueous layer was collected and transferred
206 to a new tube. RNase A (0.1mg; Lucigen) was added. Isopropanol (4°C, 1:1 volume ratio) and
207 sodium acetate (4°C, 3M, 1:10 volume ratio) were added to the mixture prior to precipitation
208 (-20°C, 1 h). The sample was then centrifuged (10000 xg, 10 mins, 4°C) before the supernatant
209 was decanted. The pellet was washed in 250µL 75% EtOH prior to centrifugation (10000 xg,
210 10 mins, 4°C). The supernatant was again decanted, and the pellet was left to air-dry, then
211 resuspended in nuclease-free $H_2O$ (30µL).

212

213 ## Genotyping for *ACE2* rs2074192

214 Genotyping was conducted by the Australian Genome Research Facility (AGRF) using the
215 Sequenom MassARRAY system, as per Zhou, *et al.*[23].

216

217 ## RNA Extraction

218 Culture media was aspirated from organoid wells and organoids were gently washed with
219 phosphate-buffered saline (PBS). Tissue was disrupted by homogenizing for 3.5 mins at 30 Hz
220 (TissueLyser, QIAGEN) in 600µL Buffer RLT Plus (RNeasy Plus Mini Kit; QIAGEN, Victoria,
221 Australia). Total RNA was extracted from the supernatant using TRIzol reagent as per
222 manufacturer's instructions and as outlined by Rio, *et al.*[24]. The purity and integrity of extracted
223 RNA samples were determined using the NanoDrop Spectrophotometer (Thermo Fisher
224 Scientific) and samples used had 260:280 and 260:230nm ratios greater than 1.9.

225

226 ## cDNA Synthesis and Quantitative Polymerase Chain Reaction (qPCR)

227 As previously described[25], synthesis of complementary DNA (cDNA) was conducted
228 beginning with 1µg of total RNA using the QuantiNova Reverse Transcription Kit (QIAGEN)
229 according to the manufacturer's protocol. qPCR was conducted with SYBR Green (QIAGEN)
230 according to manufacturer's instructions, with YWHAZ (*YWHAZ*) and β-actin (*ACTINB*) as
231 housekeeping genes (for primer sequences, see Table 2). Denaturation was performed at 95°C
232 for 10 secs, annealing at 53°C for 45 secs, and extension at 72°C for 30 secs, for a total of 50
233 cycles. qPCR results were analysed using the $2^{-\Delta\Delta CT}$ method[26].

234 ***Table 2. Sequences for qPCR primers.***

| Gene | Primer sequence (forward) | Primer sequence (reverse) |
|---|---|---|
| *ACE2* | CAGGGAACAGGTAGAGGACATT | CAGAGGGTGAACATACAGTTGG |
| *ACTINB* | CACCATTGGCAATGAGCGGTTC | AGGTCTTTGCGGATGTCCACGT |
| *YWHAZ* | ACCGTTACTTGGCTGAGGTTGC | CCCAGTCTGATAGGATGTGTTGG |

235

236 ## Protein extraction

237 Total protein was extracted from gene-edited placental organoids using a
238 radioimmunoprecipitation assay (RIPA) lysis and extraction buffer. Organoids were harvested
239 from culture wells and each sample was placed in 400µL ice cold RIPA buffer with added
240 Complete Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics Australia) and
241 phenylmethylsulfonyl fluoride (0.4 pmol). Samples were incubated (10 mins, 4°C), vortexed,
242 centrifuged (16000 xg, 10 mins, 4°C) and supernatants collected. Protein concentration was
243 measured via a Bradford Assay using the SpectraMax iD5 (Molecular Devices) at 595 nm
244 absorbance.

245

246 ## SDS-PAGE

247 As previously described[25], samples were denatured and reduced in Laemmli 4x buffer
248 (GTX16355, GeneTex) and 8% 2-mercaptoethanol (5 mins, 95°C). Samples were loaded onto
249 either a 4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gel (4568094, Bio-Rad) or 4–
250 20% Mini-PROTEAN® TGX™ Precast Protein Gel (4561094, Bio-Rad), along with a
251 molecular weight marker (GTX49384, GeneTex). SDS-PAGE was performed (1 h, 100 V) in
252 1x Running buffer (pH 8.3) using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-
253 Rad). Stain-Free Protein gels were checked for complete protein separation using the
254 ChemiDoc Touch Imaging System (Bio-Rad).

255

256 ## Transfer

257 Protein was transferred to a PVDF membrane over 16 h (27V, 4°C) in transfer buffer (25 mM
258 Trizma Base, 190 mM glycine, 20% methanol; pH 8.3) using the Criterion™ blotter (Bio-Rad).
259 Successful transfer of protein was checked by Ponceau S staining of the membrane, as well as

260 either Coomassie Blue staining (for the TGX Precast Protein Gel) or the ChemiDoc Touch
261 Imaging System (Bio-Rad) (for the TGX Stain-Free™ Protein Gel).

262

263 ## Blotting

264 Membrane was blocked in Tris Buffered Saline Tween20 (TBST) with 5% skim milk (2 h,
265 25°C). Membrane was incubated in blocking buffer (1 h, 25°C) with Anti-ACE2 Polyclonal
266 Antibody (ab15348, Abcam) at 1:300 dilution, then washed 3-4 times with TBST, and
267 incubated with a goat anti-rabbit Immunoglobulin/HRP secondary antibody (P044801-2,
268 Dako) at 1:5000 in the blocking buffer. Membrane was washed 3-4 times in TBST, then
269 incubated in Clarity Western ECL Substrate (1705060, Bio-Rad) for 5 mins and imaged. The
270 density of each band (determined by the ChemiDoc Touch Imaging System) was determined
271 prior to analysis using ImageLab software from Bio-Rad™. Analysis controlled protein loading
272 for each sample by normalizing using stain-free total protein quantification[27] and was further
273 normalized to an internal control sample (pooled TSCs) on each membrane. Samples were run
274 in duplicate and averaged for final analysis.

275

276 ## Enzyme-Linked Immunosorbent Assay (ELISA)

277 A commercially available ELISA was used to measure the concentration of ACE (DY929,
278 Duoset R&D Systems) according to manufacturer's instructions, using methods previously
279 described[28].

280

281 ## ACE2 activity assay

282 ACE2 enzyme activity was assessed according to Xiao and Burns[29] and Tamanna, *et al.*[1].
283 Briefly, samples (15µL; cell lysate or cell media) were diluted with enzyme buffer (1M NaCl,

284 75mM Tris-HCl, 5mM $ZnCl_2$; pH 6.5) with protease inhibitors (10µM captopril, 5µM
285 amastatin, 10µM bestatin; Sigma Aldrich, 10µM Z-prolyl-prolinal; Enzo Life Sciences). MCA-
286 Ala-Pro-Lys(Dnp)-OH (AnaSpec, #AS-60757), an ACE-2 specific fluorescent substrate, was
287 diluted in enzyme buffer and added to samples (final concentration 50µM). Samples were run
288 in duplicate along with a blank control in a 96-well black microplate. The plate was covered
289 and incubated on a plate shaker (24 h, 25°C).
290
291 The ACE2 standard curve was generated using human recombinant ACE2 protein (R&D
292 Systems, #933-ZN). For each concentration of ACE2 standard, two wells were dedicated to
293 measuring total ACE2 activity and two dedicated to measuring activity in the presence of
294 DX600 (AnaSpec, #AS-62337), an ACE2 inhibitor. Relative Fluorescence Units (RFU)
295 measured the product MCA-Ala-Pro over 24 h from the substrate MCA-Ala-Pro-Lys(Dnp)-
296 OH as cleaved by ACE2. Fluorescence readings (CLARIOstar Plus, BMG LabTech) were
297 taken with an excitation wavelength of 320nM, emission wavelength of 405nM.
298
299 ACE2 activity was calculated by comparing the RFU of known concentrations of ACE2
300 standard to the RFU of our samples. As such, ACE2 activity is reported as activity/ng/mL of
301 ACE2.
302

303 ## Measurement of organoid diameter and assessment of growth

304 Images of organoid wells were captured using Brightfield microscopy (Olympus Life Science).
305 All wells were imaged in the same orientation. Diameters were measured using QPath Software
306 horizontally across each organoid for n=100 organoids per group, per patient. Symmetrical
307 growth was assessed by comparing the horizontal diameter for each organoid with the vertical
308 diameter for each organoid, for n=100 organoids per group, per patient.

## Statistical Analysis

Statistical analysis for differences between groups was undertaken using SPSS Statistics Software. Outliers were removed from the data using a Grubbs' test. Data were assessed for normal distribution and a two-way ANOVA test was conducted. Differences between groups were considered significant for $p<0.05$. All tests of statistical significance are two-sided, and adjustments were made for multiple comparisons.

317 # Results

318 ## *ACE2* knockout (KO) placental organoids

319 *ACE2*$^{+/-}$ *and ACE2*$^{-/-}$ (hereafter referred to as '*ACE2* KO') placental organoids were
320 successfully generated. *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids expressed significantly more *ACE2*
321 mRNA than *ACE2* KO organoids ($p$=0.00155, $p$=0.0324 respectively; **Figure 3A**). No
322 significant difference in *ACE2* mRNA abundance was detected between *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$
323 organoids. ACE2 protein was not detected in cell media nor cell lysate of *ACE2* KO organoids
324 (nil detected). Therefore, ACE2 protein was significantly increased in cell media (**Figure 3B**)
325 and cell lysate (**Figure 3C**) of *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids compared to *ACE2* KO
326 organoids ($p$<0.0001 for all). No significant difference in ACE2 protein abundance was
327 detected between *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids in cell media or cell lysate.

328

329 ## ACE2 knockout leads to slower cell growth and cell death in placental organoids

330 *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs reached confluence at an average of 48.5 and 48.3 hours post
331 seeding, respectively. In contrast, *ACE2* KO TSCs reached confluence much later at an average
332 of 71.1 hours post seeding. *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs reached confluence significantly faster
333 than *ACE2* KO TSCs ($p$<0.0001 for all, **Figure 3D**).
334 *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs had average cell death proportions of 17% and 19%, respectively.
335 In contrast, *ACE2* KO TSCs had an average cell death proportion of 44%. *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$
336 TSCs had significantly lower proportions of cell death than *ACE2* KO TSCs ($p$<0.0001 for all,
337 **Figure 3E**).

338

339 ### *ACE2* KO markedly reduces ACE protein expression in placental organoids

340 *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ organoids expressed significantly more ACE protein than *ACE2* KO
341 organoids ($p<0.0001$, $p=0.0003$, respectively; **Figure 3F**). Given the absence of ACE2 protein
342 in *ACE2* KO organoids, the ACE2:ACE ratio was significantly higher in *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$
343 organoids compared to *ACE2* KO organoids ($p<0.0001$ for both; **Figure 3G**) but remained
344 unchanged between *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ organoids (no significant difference).

345

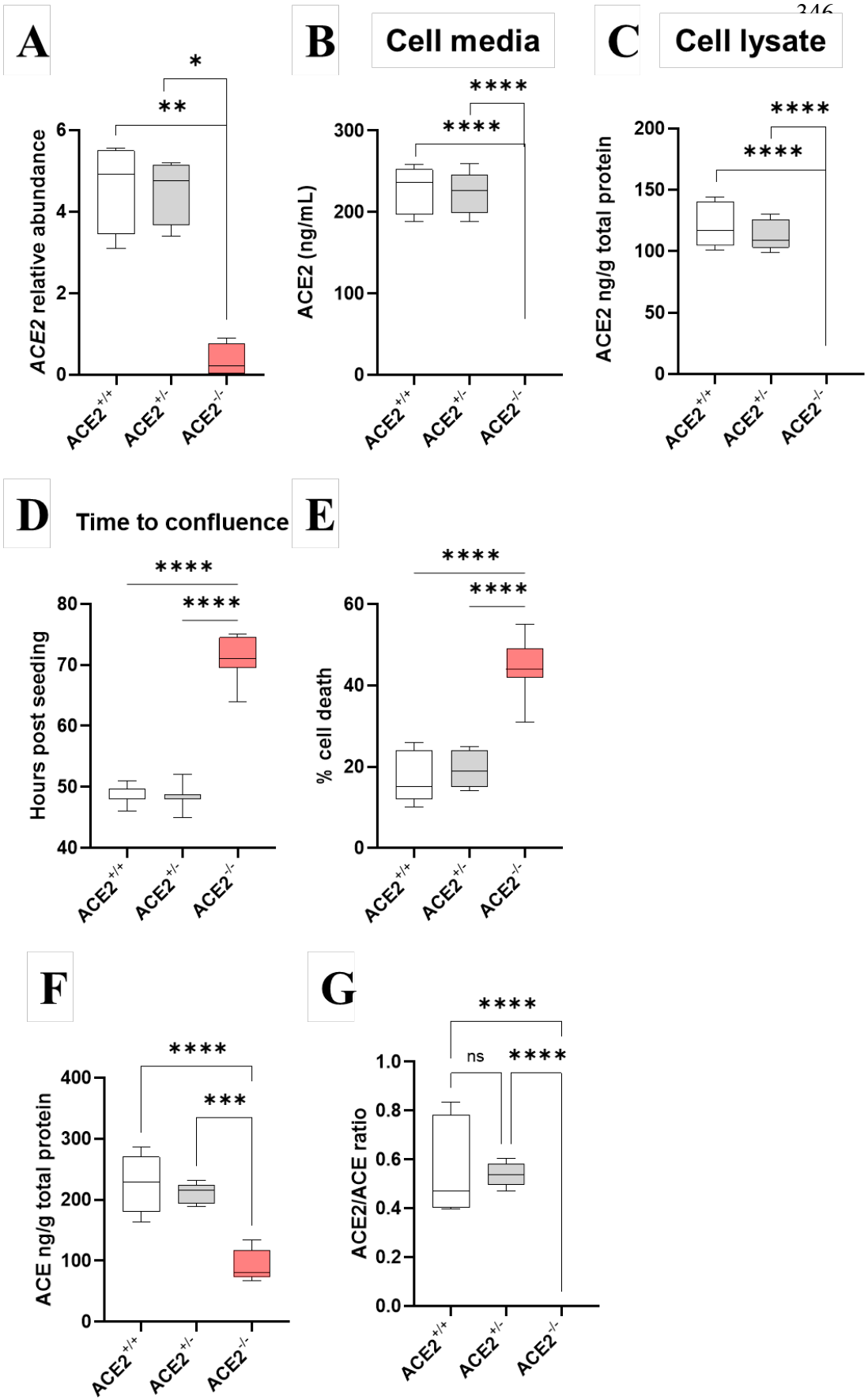
A
B
Cell media
C
Cell lysate
ACE2 relative abundance
ACE2 (ng/mL)
ACE2 ng/g total protein
D
Time to confluence
E
Hours post seeding
% cell death
F
G
ACE ng/g total protein
ACE2/ACE ratio
ns
ACE2+/+
ACE2+/-
ACE2-/-

***Figure 3.*** *The abundance of* ***(A)*** *ACE2 mRNA,* ***(B)*** *ACE2 protein in cell media and* ***(C)*** *ACE2 protein in cell lysate of* $ACE^{+/+}$*,* $ACE^{+/-}$ *and* $ACE2^{-/-}$ *(ACE2 KO) organoids.* ***(D)*** *The time to confluence, in hours post seeding, and* ***(E)*** *percentage cell death in* $ACE^{+/+}$*,* $ACE^{+/-}$ *and* $ACE2^{-/-}$ *(ACE2 KO) trophoblast stem cells (TSCs).* ***(F)*** *Level of ACE protein in cell lysate and* ***(G)*** *ACE2/ACE (ACE2:ACE) ratio (from cell lysate levels) of* $ACE^{+/+}$*,* $ACE^{+/-}$ *and* $ACE2^{-/-}$ *(ACE2 KO) organoids. Data are presented as a 10-90 percentile interleaved box-and-whisker plot. White bars denote* $ACE^{+/+}$ *group, grey bars denote* $ACE^{+/-}$ *group, red bars denote ACE2 KO group. ns indicates non-significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (n=9)*

347

348 ## *ACE2* KO reduces diameter and induces asymmetrical growth in placental organoids

349 Placental organoids tend to exhibit a spherical structure[17, 18]. However, with the *ACE2* knockout
350 we observed significantly smaller placental organoids with 'squashed', i.e. asymmetrical,
351 structures (**Figures 4A-C**). We have therefore measured the horizontal and vertical diameters
352 of organoids from each genotype to determine the effect of *ACE2* on growth and symmetry.
353
354 $ACE2^{+/+}$ and $ACE2^{+/-}$ organoids had significantly larger diameters compared to *ACE2* KO
355 organoids ($p<0.0001$ for all, **Figure 4D**).
356 Organoids were significantly less symmetrical in *ACE2* KO organoids compared with $ACE2^{+/+}$
357 organoids ($p=0.0186$, **Figure 4E**) only.
358

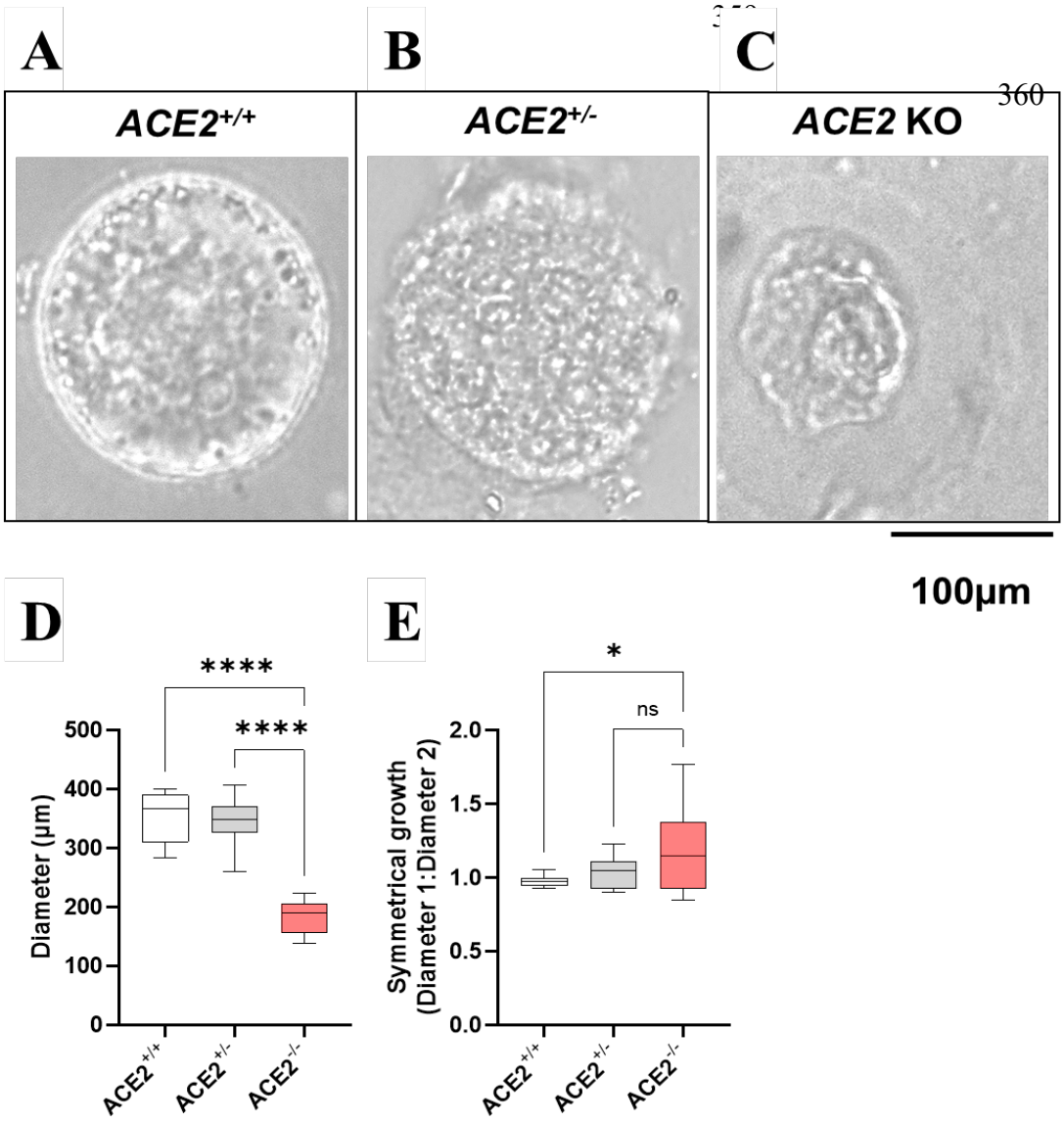


***Figure 4.*** *Representative image (Brightfield) of symmetrical organoid growth (**A**) ACE2$^{+/+}$, (**B**) ACE2$^{+/-}$ and (**C**) asymmetrical organoid growth (ACE2 KO). **(D)** The average diameter (µm) of ACE$^{+/+}$, ACE$^{+/-}$ and ACE2 KO organoids. **(E)** The ratio of diameter 1 (horizontal) to diameter 2 (vertical) of ACE$^{+/+}$, ACE$^{+/-}$ and ACE2 KO organoids as a measure of symmetrical organoid growth. A value of 1.0 indicates perfect symmetry. Data are presented as a 10-90 percentile interleaved box-and-whisker plot. White bars denote ACE$^{+/+}$ group, grey bars denote ACE$^{+/-}$ group, red bars denote ACE2 KO group. ns indicates non-significance, *p<0.05, ****p<0.0001. (n=9)*

## Placental organoids with *ACE2* rs2074192 induced SNP

TSCs were successfully gene-edited to induce the *ACE2* rs2074192 SNP. The plasmid accuracy was confirmed by Sanger sequencing (**Figure 8**), which verified a single base substitution from C to T at position 15564667 on the X chromosome (reference GRCh38.p2 38.2/144), *ACE2* gene. Transfected trophoblast stem cells were genotyped (see methods) to verify successful SNP candidates, from which organoids were created. Three genotype groups were created: TT, heterozygous SNP (CT) and homozygous SNP (TT).

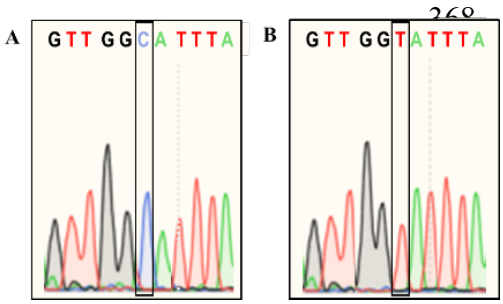


***Figure 5.*** *Successful gene-editing at position 6: the reference C allele (A) and the alternate T allele (B).*

## *ACE2* rs2074192 TT organoids, but not rs2074192 CT organoids, have increased *ACE2* mRNA and ACE2 protein

TT organoids had significantly higher expression of *ACE2* mRNA compared with both CC and CT organoids ($p$=0.0040, $p$=0.0141, respectively; **Figure 6A**). TT organoids also had significantly higher levels of ACE2 protein than CC and CT organoids in both the cell media ($p$=0.0013, $p$=0.0169, respectively; **Figure 6B**) and cell lysate ($p$=0.0018, $p$=0.0040, respectively; **Figure 6C**). There was no significant difference in abundance of *ACE2* mRNA, nor ACE2 protein in cell media or lysate, between CC and CT organoids.

Time to confluence, and percentage cell death, were also assessed, however no significant differences were detected between groups (data not shown).

384

385 ## *ACE2* rs2074192 genotype did not change ACE protein expression, but increased the
386 ACE2/ACE ratio in TT organoids

387 There was no significant difference in ACE protein level (measured in cell lysate) between CC,
388 CT and TT organoids (**Figure 6D**). However, the ACE2/ACE ratio was significantly higher in
389 TT organoids compared to both CC and CT organoids ($p$=0.0049, $p$=0.0093, respectively;
390 **Figure 6E**). There was no significant difference in ACE protein expression, nor ACE2/ACE
391 ratio, between CC and CT organoids.

392

393 ## ACE2 activity is decreased in *ACE2* rs2074192 TT organoids

394 ACE2 enzyme activity was significantly reduced in the cell media of TT organoids compared
395 to both CC and CT organoids ($p$<0.0001 for both; **Figure 6F**). There was no significant
396 difference in ACE2 activity in the cell media of CT organoids compared to CC organoids.
397 ACE2 enzyme activity was significantly reduced in the cell lysate of TT organoids compared
398 to CC organoids ($p$=0.0303; **Figure 6G**). There was no significant difference in ACE2 activity
399 in the cell lysate of CT organoids compared to TT organoids, nor compared to CC organoids.

400

401 The ratio between ACE2 activity and ACE2 levels was significantly decreased in the cell media
402 of TT organoids compared to both CC and CT organoids ($p$<0.0001 for both; **Figure 6H**), while
403 there was no significant difference between CT and CC organoids. The ratio between ACE2
404 activity and ACE2 levels was significantly decreased in the cell lysate of TT organoids
405 compared to both CC and CT organoids ($p$<0.0001 for both; **Figure 6I**), while there was no
406 significant difference in the ratio between CT and CC organoids.

407

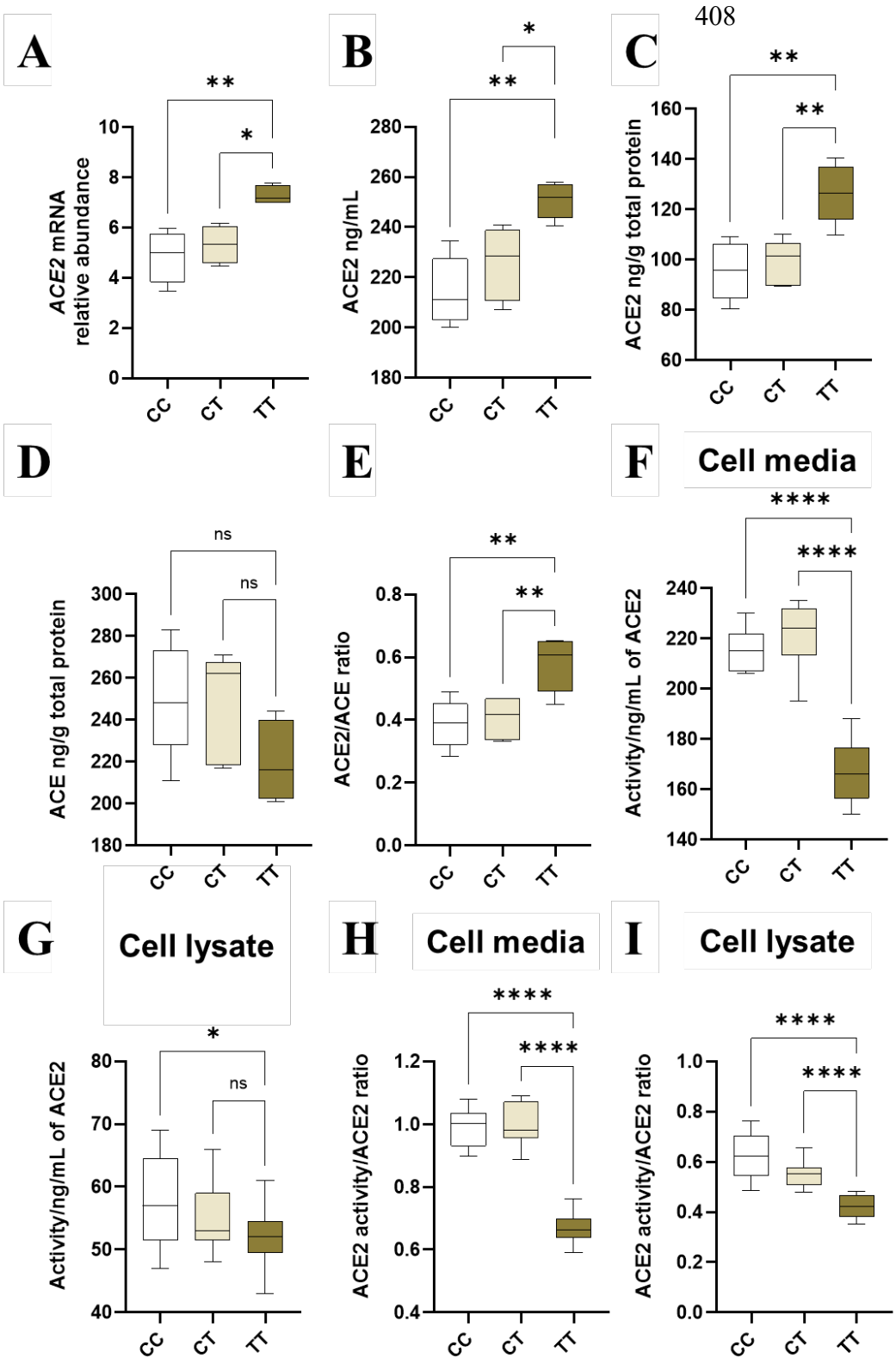
A
ACE2 mRNA relative abundance
CC
CT
TT
B
ACE2 ng/mL
C
ACE2 ng/g total protein
D
ACE ng/g total protein
ns
E
ACE2/ACE ratio
F
Cell media
Activity/ng/mL of ACE2
G
Cell lysate
H
Cell media
ACE2 activity/ACE2 ratio
I
Cell lysate

***Figure 6.*** *The abundance of* ***(A)*** *ACE2 mRNA,* ***(B)*** *ACE2 protein in cell media and* ***(C)*** *ACE2 protein in cell lysate;* ***(D)*** *Level of ACE protein in cell lysate,* ***(E)*** *ACE2/ACE ratio (from cell lysate levels); Enzyme activity of ACE2 in* ***(F)*** *cell media and (****G****) cell lysate, and ratio between ACE2 activity and ACE2 levels in (****H****) cell media and (****I****) cell lysate of CC, CT and TT organoids. Data are presented as a 10-90 percentile interleaved box-and-whisker plot. White bars denote CC group, beige bars denote CT group, brown bars denote TT group. ns indicates non-significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (n=9)*

409

## *ACE2* rs2074192 TT organoids have reduced diameters and asymmetrical growth

The diameter of TT organoids was significantly reduced compared to CC organoids ($p$=0.0141; **Figure 7A**) only. Organoid growth was significantly less symmetrical in TT organoids compared to both CC and CT organoids ($p$=0.0077, $p$=0.0415, respectively; **Figure 7B**). There was no significant difference in diameter or symmetrical growth measurements between CC and CT organoids.

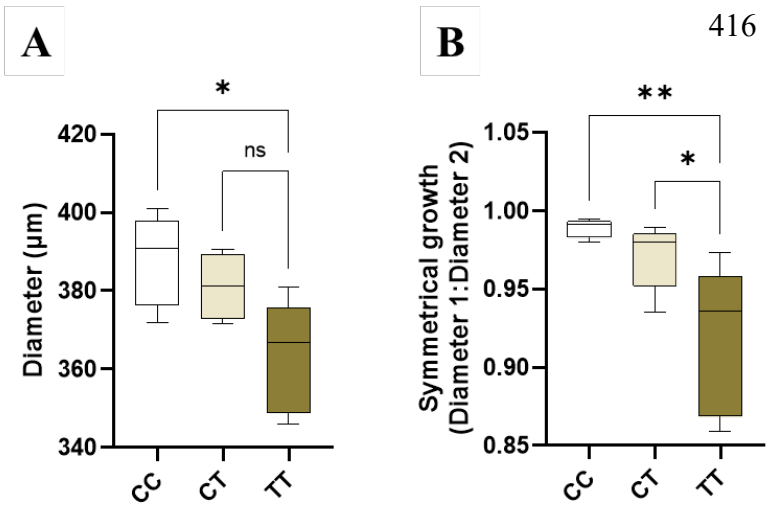


***Figure 7. (A)*** *The average diameter (µm) of CC, CT and TT organoids.* ***(B)*** *The ratio of diameter 1 (horizontal) to diameter 2 (vertical) of CC, CT and TT organoids as a measure of symmetrical organoid growth. A value of 1.0 indicates perfect symmetry. Data are presented as a 10-90 percentile interleaved box-and-whisker plot. White bars denote CC group, beige bars denote CT group, brown bars denote TT group. ns indicates non-significance, *p<0.05, **p<0.01. (n=9)*

417 ## Discussion

418 This study is the first to successfully use gene-editing to stably produce *ACE2* knockout
419 placental organoids, and the first to induce a SNP in placental organoids. Using these models,
420 the study demonstrates that *ACE2* is important for cell growth (as evidenced by changes in the
421 time to confluence and percentage cell death of TSCs), as well as placental organoid size and
422 growth (as evidenced by changes in organoid diameters and symmetry growth measurements).
423 Furthermore, we show that ACE2:ACE ratio is disrupted in both organoid models, resulting in
424 impaired growth and development. Our data are consistent with our hypothesis that *ACE2* plays
425 an important role in placental development, and that ACE2 and ACE need to exist in a finely
426 tuned balance to ensure proper growth and morphogenesis. We have also confirmed that the
427 *ACE2* rs2074192 TT genotype results in increased ACE2 mRNA and protein, and altered
428 ACE2:ACE ratios, which likely mediate the effects on TSC and organoids growth and cell
429 survival.
430
431 *ACE2* knockout mouse models of pregnancy have been useful in reinforcing the importance of
432 ACE2 in placentation and pregnancy health. This study has extended our understanding of the
433 role of ACE2 in the placenta, creating the first *ACE2* knockout in primary human placenta cells.
434 Furthermore, we have explored the molecular implications of carriage of a common *ACE2*
435 SNP, rs2074192. *ACE2* variants are associated with pregnancy complications, as well as with
436 a number of other diseases characterised by renin-angiotensin system dysfunction, including
437 COVID-19[13,14], hypertension[12, 30] and heart failure[15]. *ACE2* rs2074192 is a common SNP, with
438 an estimated frequency of C (reference allele) = 0.63 and T (alternate allele) = 0.37, according
439 to a study of 102,615 people (including varied ethnicities) in the Genome Aggregation
440 Database[16]. This suggests that at least one copy of this SNP naturally occurs in over one third
441 of the population, and a recent study indicated that the incidence of the TT genotype in their

442 study population was 19.8%[10]. This highlights the importance of understanding the mechanism
443 by which carriage of the alternate allele increases the risk of disease.
444
445 *In silico* analysis of *ACE2* rs2074192 suggests that it increases splicing donor sites, creating a
446 changed secondary RNA structure, affecting protein production and activity. As *ACE2*
447 rs2074192 is an intronic polymorphism, it is likely that it indirectly positively regulates gene
448 expression by altering ACE2 splicing and transcription enhancement[13]. This study confirms
449 that carriage of the TT genotype, but not the CT genotype, results in increased placental ACE2
450 mRNA and protein expression. This has major implications for the pathogenesis of diseases
451 dependent on ACE2 expression, including pregnancy complications such as preeclampsia[1] and
452 SGA[1], and infection with SARS-CoV-2 virus[31], which can directly infect syncytiotrophoblasts
453 through earlier endometrial cell infection[32]. However, the mechanism of action by which this
454 SNP contributes to pathogenesis requires further study. Both the *ACE2* rs2074192 (C to T) SNP
455 and haplotypes CAGC and TAGT are associated with altered levels of the Ang 1-7 peptide, a
456 direct product of ACE2 activity[10]. As this study did not quantify Ang 1-7 expression, further
457 studies should investigate whether Ang 1-7 is altered as a result of *ACE2* rs2074192 genotype,
458 providing additional information on a potential mechanism for pathogenesis of diseases
459 dependent on ACE2 in T allele carriers.
460
461 This study observed that *ACE2* knockout TSCs took longer to reach confluence and had higher
462 percentages of cell death in culture. This finding has implications for pregnancy complications
463 with origins in early gestation, when TSCs play an important role in placental development
464 that underpins subsequent function. During early gestation, the placenta undergoes rapid
465 trophoblast proliferation and differentiation[33]. Not only did this study observe slower TSC
466 growth and higher levels of cell death in *ACE2* knockouts, but *ACE2* knockout organoids had

467 reduced diameters, as well as asymmetrical growth, reflecting impaired organoid development.
468 Interestingly, although no change in TSC growth or cell death was observed with *ACE2*
469 rs2074192 genotype, TT organoids also had smaller diameters and asymmetrical growth.
470
471 This impaired growth has concerning implications for placental development. Human placental
472 organoids provide an innovative model for studying placental development *in vitro*. Ethical
473 limitations to study of early- to mid-gestation placentae, created the need for physiologically
474 relevant human placental models with 3D architecture. Current models are limited[34, 35, 36] while
475 mouse placenta differs both in structure and endocrine function[37]. Organoids recapitulate all
476 first-trimester placental functions, can differentiate into the expected cell lineages, and are able
477 to be cultured long-term without the loss of these features[38]. For this reason, human placental
478 organoids are the closest possible parallel to studying the early gestation human placenta *in*
479 *vivo*. *ACE2* gene-editing of placental organoids in this study provides insight into the
480 detrimental effect of *ACE2* knockout and rs2074192 TT genotype on placental development,
481 likely leading to aberrant placentation in human pregnancy.
482
483 Whilst an absence of ACE2 is clearly detrimental to cell growth and increases cell death, a
484 complete knockout is highly unlikely in nature and thus is less biologically relevant for causal
485 inference. Therefore, we have compared the knockout and induced SNP models to make
486 inferences most biologically relevant to human pregnancy. Surprisingly, both the *ACE2* KO
487 organoids and the induced SNP organoids exhibited diminished growth and symmetry, despite
488 *ACE2* KO organoids having markedly reduced ACE2 protein, and induced SNP organoids
489 having markedly increased protein, compared to their respective controls. This result suggests
490 that the absolute expression value of ACE2 is the not sole determinant of organoid growth.
491

492 One common feature between *ACE2* knockout organoids and TT organoids is the altered
493 ACE2:ACE ratio. Our data suggest that ACE2 and ACE, which are known to partly control cell
494 growth[3], have more contributions to placental organoid development (as evidenced by organoid
495 symmetry and growth) than simply by affecting cell proliferation and death. This is evident
496 from our observation of unchanged TSC growth and cell death levels in TT TSCs, despite them
497 also having altered ACE2 and ACE expression.
498
499 The mechanism for this unexpected increase in ACE2 protein, but decreased organoid growth
500 and symmetry, could be due to the reduced ACE2 enzyme activity observed in TT organoids.
501 *ACE2* rs2074192 is an intronic SNP, which does not directly impact protein coding regions of
502 the genome. Therefore, the contribution of this SNP to ACE2 protein increase is indirect, likely
503 through a complex mechanism not yet elucidated. We hypothesise that the excess of ACE2
504 protein in TT organoids has resulted in a compensatory response in the cell, reducing the
505 *activity* of ACE2 in an attempt to re-establish an ACE2:ACE equilibrium. This theory is
506 supported by the literature, showing that *ACE2* rs2074192 is associated with increased risk of
507 SGA[5], a condition in which inadequate placentation often leads to smaller placentae and a
508 smaller fetus. Furthermore, in SGA, ACE2 activity is significantly reduced[1].
509
510 This study indicates that, whilst ACE2 expression is significant, the activity of the enzyme and
511 maintenance of the ACE2 and ACE in equilibrium (in this paper termed an ACE2:ACE ratio)
512 may be of greater importance. Studies investigating pregnancy complications support this
513 conclusion. In preeclampsia, a potentially life-threatening complication characterised by
514 maternal hypertension and multi-organ failure, ACE2 activity reflected by the ACE2:ACE ratio
515 is higher than normal, impairing placental development and function[1]. In SGA, placental
516 expression of ACE and ACE2 at delivery are both higher compared with uncomplicated

517 pregnancy. Birthweight centiles are negatively correlated with maternal plasma ACE and ACE2
518 levels in mid-pregnancy, suggesting that tight regulation of these enzymes is critical to ensure
519 pregnancy health[1]. As previously mentioned, the ratio of ACE2 activity to ACE2 levels in
520 maternal plasma is significantly reduced in SGA, indicating that ACE2 operates with reduced
521 efficiency in this condition[1]. This could be due to the increased presence of an endogenous
522 inhibitor[39] in SGA, although this has never been investigated. Placental ACE2 deficiency, and
523 the associated elevation in placental Ang II levels, negatively impact pregnancy by impairing
524 maternal weight gain and restricting fetal growth[2]. This implies the balance of ACE2:ACE
525 supersedes the importance of ACE2 levels in isolation in determining fetal growth. Indeed,
526 herein we observed similar reductions in organoid diameter and growth between the complete
527 *ACE2* knockout and rs2074192 models, despite the difference in absolute ACE2 expression
528 values.
529
530 Interestingly, this study showed that *ACE2* rs2074192 TT organoids not only had elevated
531 ACE2 protein within cells (as assessed by cell lysate protein levels) but also secreted more
532 ACE2 protein into the cell media. Whilst ACE2 is typically a membrane-bound peptidase, it
533 can also be shed from the membrane[40], producing soluble ACE2 (sACE2). It should be noted
534 that this study utilised only an ACE2 antibody specific to the membrane-bound ACE2 protein
535 and we cannot comment on the production of sACE2 by these organoid models. Future studies
536 will utilise a specific sACE2 antibody to examine this.
537
538 A limitation of this study is that it only utilised placental tissue from female fetuses. This is
539 because placental expression of ACE and ACE2 is fetal sex dependent. Both ACE and ACE2
540 are more highly expressed in placentae from female compared to male fetuses[41]. For ACE2,
541 this is likely due to the fact that *ACE2*, located on the X-chromosome, escapes X chromosome

542 inactivation, thus often two copies are expressed instead of one in females[42]. The balance of
543 downstream effector peptides Ang II (via ACE) and Ang 1-7 (via ACE2) is also influenced by
544 fetal sex. In early gestation, Ang 1-7:Ang II ratios are reduced in placenta from females,
545 indicating less Ang 1-7 production as a result of reduced ACE2 activity[43]. Female babies are
546 more likely to be growth restricted than males[44, 45]. In this study, the organoid growth symmetry
547 only significantly changed between the *ACE2*$^{+/+}$ and total knockout, but not *ACE2*$^{+/-}$ and the
548 knockout. This could indicate that in males, where only one *ACE2* gene is present (due to the
549 presence of only one X chromosome), there may be differences in placental development. Thus,
550 *ACE2* fetal sex-specific molecular roles require further investigation. Future studies should
551 include male samples, as well as females to expand on the findings from this study.
552
553 Our data clearly demonstrate that ACE2 plays an important role in placental organoid
554 development, and its expression is increased with the rs2074192 TT genotype. Importantly,
555 ACE2 enzyme activity and the ACE2:ACE ratio appeared to make more difference to the
556 growth and development of TSCs and placental organoids than the absolute expression values
557 of ACE2 alone. We anticipate that this study will be used to facilitate a deeper understanding
558 of the role of ACE2 in placental development. The gene-editing technique in placental
559 organoids that we have developed could be used to study the roles of many other genes and
560 their variants on placental growth and function.
561

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## Author contributions

A.L.A conceptualised this work, performed experimental work and formal analysis of data, original draft preparation, and review and editing. B.D. and M.K. provided valuable contributions to methodology optimisation, and reviewed and edited the manuscript. C.J.L., P.Q.T. and F.A. designed and generated plasmids used for gene-editing, and reviewed and edited the manuscript. T.J-K., M.D.S. and K.G.P. reviewed and edited the manuscript. C.T.R. provided supervision, funding acquisition, interpretation of data, review and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of Interest

The authors declare no conflict of interest.

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