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

Anya L. Arthurs*[1], Bianca Dietrich[2], Martin Knöfler[2], Caleb J. Lushington[3,4], Paul Q. Thomas[3,4,5], Fatwa Adikusuma[3,4], Tanja Jankovic-Karasoulos[1], Melanie D. Smith[1], Kirsty G. Pringle[6,7], Claire T. Roberts[1]

[1] Flinders University, College of Medicine and Public Health, Flinders Health and Medical Research Institute, Adelaide, South Australia, Australia

[2] Placental Development Group, Medical University of Vienna, A-1090 Vienna, Austria

[3] University of Adelaide, School of Biomedicine and Robinson Research Institute, Adelaide, South Australia, Australia

[4] South Australian Health and Medical Research Institute (SAHMRI), Genome Editing Program, Adelaide, South Australia, Australia

[5] South Australian Health and Medical Research Institute (SAHMRI), South Australian Genome Editing (SAGE), Adelaide, SA, Australia

[6] School of Biomedical Sciences and Pharmacy, College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, New South Wales, Australia

[7] Mothers and Babies Research Program, Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia

*Corresponding author: anya.arthurs@flinders.edu.au

ORCID: https://orcid.org/0000-0002-4382-0610

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

Introduction

Understanding the molecular mechanisms underlying pregnancy and fetal development is crucial for improving maternal and neonatal health outcomes. Despite significant advances in obstetric care, complications such as small-for-gestational-age, fetal growth restriction and preeclampsia continue to pose substantial risks. Recent studies have highlighted the pivotal role of placental function in these conditions, with literature suggesting that variations in placental angiotensin converting enzyme 2 (*ACE2*) gene expression may be a key factor. However, there remains a gap in our comprehensive understanding of how *ACE2* genetic variations and molecular pathways contribute to these pregnancy disorders.

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

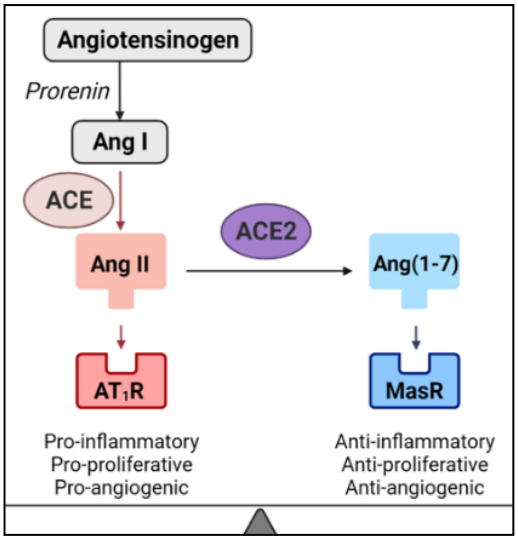

Figure 1. *The ACE- and ACE2-mediated opposing arms of the renin-angiotensin system.*

The circulating renin-angiotensin system is a critical regulator of blood volume and systemic vascular resistance. However, in addition to the circulatory system, local tissue-specific renin-angiotensin systems exist, including within the placenta. The pro-inflammatory, pro-proliferative arm of the placental renin-angiotensin system (shown in red in **Figure 1**), mediated by ACE, promotes cell and tissue growth. This is opposed by an anti-inflammatory, anti-proliferative arm (shown in blue in **Figure 1**), mediated by ACE2[3]. ACE and ACE2 maintain a finely tuned balance within the renin-angiotensin system to optimise placental growth and function throughout healthy pregnancy. However, the expression and activity of ACE and ACE2 enzymes, both systemically[1, 2] and in the placenta[3, 4, 5, 6, 7], is disturbed in many pregnancy complications associated with renin-angiotensin system dysfunction.

Adequate ACE2 expression and activity, in both the mother and placenta, is necessary for healthy pregnancy. In *ACE2*$^{-/-}$(*ACE2* knockout) mouse models, *ACE2* knockout dams have higher systolic blood pressure[2, 4], decreased plasma Ang 1-7[2], and placentae have increased levels of[2], and sensitivity to[4], Ang II. This results in alterations to the balance of ACE and ACE2 activity in the renin-angiotensin system. Furthermore, pups from *ACE2* knockout dams have reduced weight[2, 4] and length[2], with lower pup to placenta weight ratios, indicating compromised placental function. Clearly ACE2 is essential for healthy pregnancy but dysregulation of ACE2 levels and activity, as well as ACE:ACE2 balance, is pathogenic[4, 11]. However, whilst murine research is informative, an *ACE2* knockout model of human placenta has never been established.

As previously mentioned, *ACE2* gene variants (including single nucleotide polymorphisms [SNPs]) alter expression of renin-angiotensin system components. A SNP is a single base

substitution at a specific chromosome location. Many SNPs in the *ACE2* gene have been associated with disease states[5, 8, 12, 13, 14]. In particular, *ACE2* rs2074192 is associated with increased incidence of diseases characterised by renin-angiotensin system dysregulation[12, 13, 14, 15], including an increased susceptibility to COVID-19 and increased disease severity[13, 14]. Importantly for pregnancy, SGA, a condition where neonatal birthweight is lower than expected for their gestational age, has been associated with *ACE2* rs2074192 (C to T polymorphism) which, when carried by the fetus, increases the risk of SGA by 23-fold[5]. The placenta, a product of conception, is genetically identical to the fetus. As such, a fetal SNP will also be present in the placenta. Despite strong associations between *ACE2* rs2074192 frequency and disease state, and the estimated frequency of at least one copy of this SNP in one third of the global population[16], research is mostly limited to association studies but not its mechanisms of action.

In this study, we utilised gene-editing to generate *ACE2* knockout placental organoids and *ACE2* rs2074192 placental organoids, creating the first induced SNP gene-edited organoids in human placenta. We investigated the role of *ACE2* in placental development by profiling expression of *ACE2* mRNA and protein, ACE protein, ACE2 enzyme activity and parameters of cell and organoid growth in both placental organoid models. Elucidating the role of *ACE2* in the placenta will allow inferences to be made regarding its overall contribution to pregnancy health, as well as its possible role in other physiological and pathological states that involve the renin-angiotensin system.

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**).

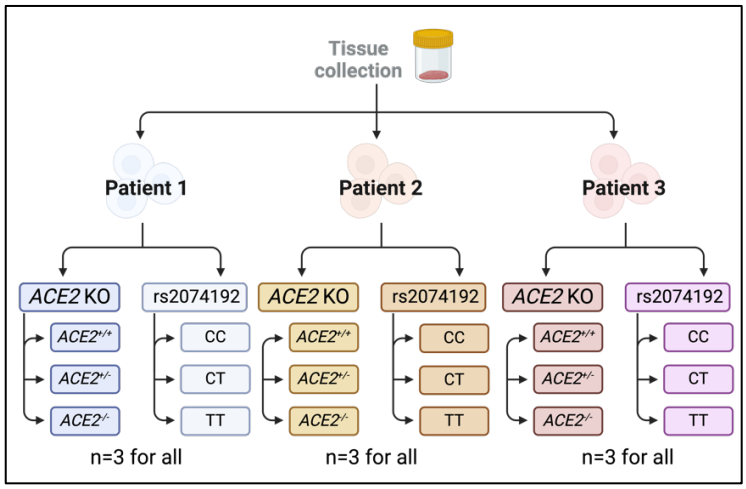

***Figure 2**. Method describing gene-editing to generate different TSC groups.*

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

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

141

142 ## Genotyping for fetal sex

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

155

156 ## Isolation and cultivation of TSCs

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

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

167 Generation of plasmids

168 To generate modified ACE2 organoid models, two distinct gene editing strategies were
169 employed. Firstly, a modified pDG459 plasmid (Addgene #100901) was designed to induce a
170 complete *ACE2* knockout (KO). This entailed the precise targeting of regions approximately
171 100 base pairs upstream and downstream of the first and last *ACE2* exons, resulting in the
172 excision of promoter, regulatory, and coding sequences. Secondly, a modified PEA1-Puro
173 plasmid (Addgene #171991) was designed to introduce an intronic *ACE2* single nucleotide
174 polymorphism (SNP), specifically rs2074192. The SNP was targeted using the PE3 strategy,
175 which incorporated a downstream nicking single-guide RNA (sgRNA) positioned 72 base pairs
176 from the primary cleavage site, adhering to the recommended range of 40-90 nucleotides[19].
177 Construct assembly was achieved through *BbsI*-mediated golden gate assembly of
178 phosphorylated and annealed oligonucleotide guide sequences (Thermo Fisher), following
179 optimised protocols for PEA1 or pDG459 assembly[20, 21]. Subsequent plasmid purification was
180 performed using the QIAprep Spin Miniprep Kit (Qiagen), and validation of oligonucleotide
181 insertion was carried out through Sanger sequencing (AGRF). Oligonucleotide sequences are
182 listed in **Table 1**.
183

184 ***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

185

186 ## TSC genetic modification

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

195

196 ## Assessment of Time to Confluence and Cell Death

197 Immediately after culture with puromycin, gene-edited TSCs were passaged using TrypLE
198 Reagent (TrypLE, Gibco), according to manufacturer's instructions, then replated at a density

199 of ~20% (1:5 dilution) per well. Cells were then grown in the Incucyte® SX5 for 4 days (until
200 confluence). Cell confluence percentage and cell death were assessed using Incucyte analysis
201 software. Once cells reached confluence, TSCs were removed from the Incucyte and organoids
202 were created.

203

204 ## Organoid formation

205 Placental organoids were established as per Haider, *et al.*[18], with minor modifications. Briefly,
206 gene-edited TSCs were trypsinised (TrypLE, Gibco) and washed in ice-cold advanced
207 Dulbecco's Modified Eagle Medium (DMEM, Gibco). Cells were resuspended in organoid
208 media: ice-cold advanced DMEM supplemented with 1×B-27, 1×ITS-X, 10 mM HEPES
209 (Gibco), 2 mM glutamine (Gibco), 1 µM A8391, 100 ng/ml rhEGF and 3 µM CHIR99021.
210 Vitrogel (ORGANOID-1, The Well BioScience) was added to reach a 2:1 Vitrogel to organoid
211 media ratio. 75µL of the resultant mixture was plated in a 6-well culture plate, forming a dome
212 in the centre of the well. After incubation (37°C, 2 h), 1.5mL organoid media (37°C) was added
213 to each well. Media was changed every 5 days until organoids reached confluence, at which
214 point they were harvested for RNA and protein experiments.

215

216 ## Genomic DNA (gDNA) extraction

217 gDNA extraction protocol was adapted from Ghatak, *et al.*[22]. Prior to organoid generation, a
218 subset of TSCs from each gene-edited patient sample was harvested and gDNA was extracted
219 by incubating cells with a phenol:chloroform:isoamyl alcohol solution (25:24:1). After
220 centrifugation (10 mins, 10000 xg, 4°C), the upper aqueous layer was collected and transferred
221 to a new tube. RNase A (0.1mg; Lucigen) was added. Isopropanol (4°C, 1:1 volume ratio) and
222 sodium acetate (4°C, 3M, 1:10 volume ratio) were added to the mixture prior to precipitation

223 (-20°C, 1 h). The sample was then centrifuged (10000 xg, 10 mins, 4°C) before the supernatant
224 was decanted. The pellet was washed in 250µL 75% EtOH prior to centrifugation (10000 xg,
225 10 mins, 4°C). The supernatant was again decanted, and the pellet was left to air-dry, then
226 resuspended in nuclease-free H_2O (30µL).

227

228 ## Genotyping for *ACE2* rs2074192

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

231

232 ## RNA Extraction

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

240

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

242 As previously described[25], synthesis of complementary DNA (cDNA) was conducted
243 beginning with 1µg of total RNA using the QuantiNova Reverse Transcription Kit (QIAGEN)
244 according to the manufacturer's protocol. qPCR was conducted with SYBR Green (QIAGEN)
245 according to manufacturer's instructions, with YWHAZ (*YWHAZ*) and β-actin (*ACTINB*) as
246 housekeeping genes (for primer sequences, see Table 2). Denaturation was performed at 95°C

247 for 10 secs, annealing at 53°C for 45 secs, and extension at 72°C for 30 secs, for a total of 50
248 cycles. qPCR results were analysed using the $2^{-\Delta\Delta CT}$ method[26].

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

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

250

251 ## Protein extraction

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

260

261 ## SDS-PAGE

262 As previously described[25], samples were denatured and reduced in Laemmli 4x buffer
263 (GTX16355, GeneTex) and 8% 2-mercaptoethanol (5 mins, 95°C). Samples were loaded onto
264 either a 4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gel (4568094, Bio-Rad) or 4–
265 20% Mini-PROTEAN® TGX™ Precast Protein Gel (4561094, Bio-Rad), along with a
266 molecular weight marker (GTX49384, GeneTex). SDS-PAGE was performed (1 h, 100 V) in

267 1x Running buffer (pH 8.3) using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-
268 Rad). Stain-Free Protein gels were checked for complete protein separation using the
269 ChemiDoc Touch Imaging System (Bio-Rad).

270

271 ## Transfer

272 Protein was transferred to a PVDF membrane over 16 h (27V, 4°C) in transfer buffer (25 mM
273 Trizma Base, 190 mM glycine, 20% methanol; pH 8.3) using the Criterion™ blotter (Bio-Rad).
274 Successful transfer of protein was checked by Ponceau S staining of the membrane, as well as
275 either Coomassie Blue staining (for the TGX Precast Protein Gel) or the ChemiDoc Touch
276 Imaging System (Bio-Rad) (for the TGX Stain-Free™ Protein Gel).

277

278 ## Blotting

279 Membrane was blocked in Tris Buffered Saline Tween20 (TBST) with 5% skim milk (2 h,
280 25°C). Membrane was incubated in blocking buffer (1 h, 25°C) with Anti-ACE2 Polyclonal
281 Antibody (ab15348, Abcam) at 1:300 dilution, then washed 3-4 times with TBST, and
282 incubated with a goat anti-rabbit Immunoglobulin/HRP secondary antibody (P044801-2,
283 Dako) at 1:5000 in the blocking buffer. Membrane was washed 3-4 times in TBST, then
284 incubated in Clarity Western ECL Substrate (1705060, Bio-Rad) for 5 mins and imaged. The
285 density of each band (determined by the ChemiDoc Touch Imaging System) was determined
286 prior to analysis using ImageLab software from Bio-Rad™. Analysis controlled protein loading
287 for each sample by normalizing using stain-free total protein quantification[27] and was further
288 normalized to an internal control sample (pooled TSCs) on each membrane. Samples were run
289 in duplicate and averaged for final analysis.

290

291 ## Enzyme-Linked Immunosorbent Assay (ELISA)

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

295

296 ## ACE2 activity assay

297 ACE2 enzyme activity was assessed according to Xiao and Burns[29] and Tamanna, *et al.*[1].
298 Briefly, samples (15µL; cell lysate or cell media) were diluted with enzyme buffer (1M NaCl,
299 75mM Tris-HCl, 5mM $ZnCl_2$; pH 6.5) with protease inhibitors (10µM captopril, 5µM
300 amastatin, 10µM bestatin; Sigma Aldrich, 10µM Z-prolyl-prolinal; Enzo Life Sciences). MCA-
301 Ala-Pro-Lys(Dnp)-OH (AnaSpec, #AS-60757), an ACE-2 specific fluorescent substrate, was
302 diluted in enzyme buffer and added to samples (final concentration 50µM). Samples were run
303 in duplicate along with a blank control in a 96-well black microplate. The plate was covered
304 and incubated on a plate shaker (24 h, 25°C).

305

306 The ACE2 standard curve was generated using human recombinant ACE2 protein (R&D
307 Systems, #933-ZN). For each concentration of ACE2 standard, two wells were dedicated to
308 measuring total ACE2 activity and two dedicated to measuring activity in the presence of
309 DX600 (AnaSpec, #AS-62337), an ACE2 inhibitor. Relative Fluorescence Units (RFU)
310 measured the product MCA-Ala-Pro over 24 h from the substrate MCA-Ala-Pro-Lys(Dnp)-
311 OH as cleaved by ACE2. Fluorescence readings (CLARIOstar Plus, BMG LabTech) were
312 taken with an excitation wavelength of 320nM, emission wavelength of 405nM.

313

314 ACE2 activity was calculated by comparing the RFU of known concentrations of ACE2
315 standard to the RFU of our samples. As such, ACE2 activity is reported as activity/ng/mL of
316 ACE2.
317

318 ## Measurement of organoid diameter and assessment of growth

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

324

325 ## Statistical Analysis

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

331

332 # Results

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

334 *ACE2*$^{+/-}$ *and ACE2*$^{-/-}$ (hereafter referred to as '*ACE2* KO') placental organoids were
335 successfully generated. *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids expressed significantly more *ACE2*
336 mRNA than *ACE2* KO organoids (p=0.00155, p=0.0324 respectively; **Figure 3A**). No
337 significant difference in *ACE2* mRNA abundance was detected between *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$
338 organoids. ACE2 protein was not detected in cell media nor cell lysate of *ACE2* KO organoids
339 (nil detected). Therefore, ACE2 protein was significantly increased in cell media (**Figure 3B**)
340 and cell lysate (**Figure 3C, 3D**) of *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids compared to *ACE2* KO
341 organoids (p<0.0001 for all). No significant difference in ACE2 protein abundance was
342 detected between *ACE2*$^{+/+}$ *and ACE2*$^{+/-}$ organoids in cell media or cell lysate.

343

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

345 *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs reached confluence at an average of 48.5 and 48.3 hours post
346 seeding, respectively. In contrast, *ACE2* KO TSCs reached confluence much later at an average
347 of 71.1 hours post seeding. *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs reached confluence significantly faster
348 than *ACE2* KO TSCs (p<0.0001 for all, **Figure 3E**).
349 *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$ TSCs had average cell death proportions of 17% and 19%, respectively.
350 In contrast, *ACE2* KO TSCs had an average cell death proportion of 44%. *ACE2*$^{+/+}$ and *ACE2*$^{+/-}$
351 TSCs had significantly lower proportions of cell death than *ACE2* KO TSCs (p<0.0001 for all,
352 **Figure 3F**).

353

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

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

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361

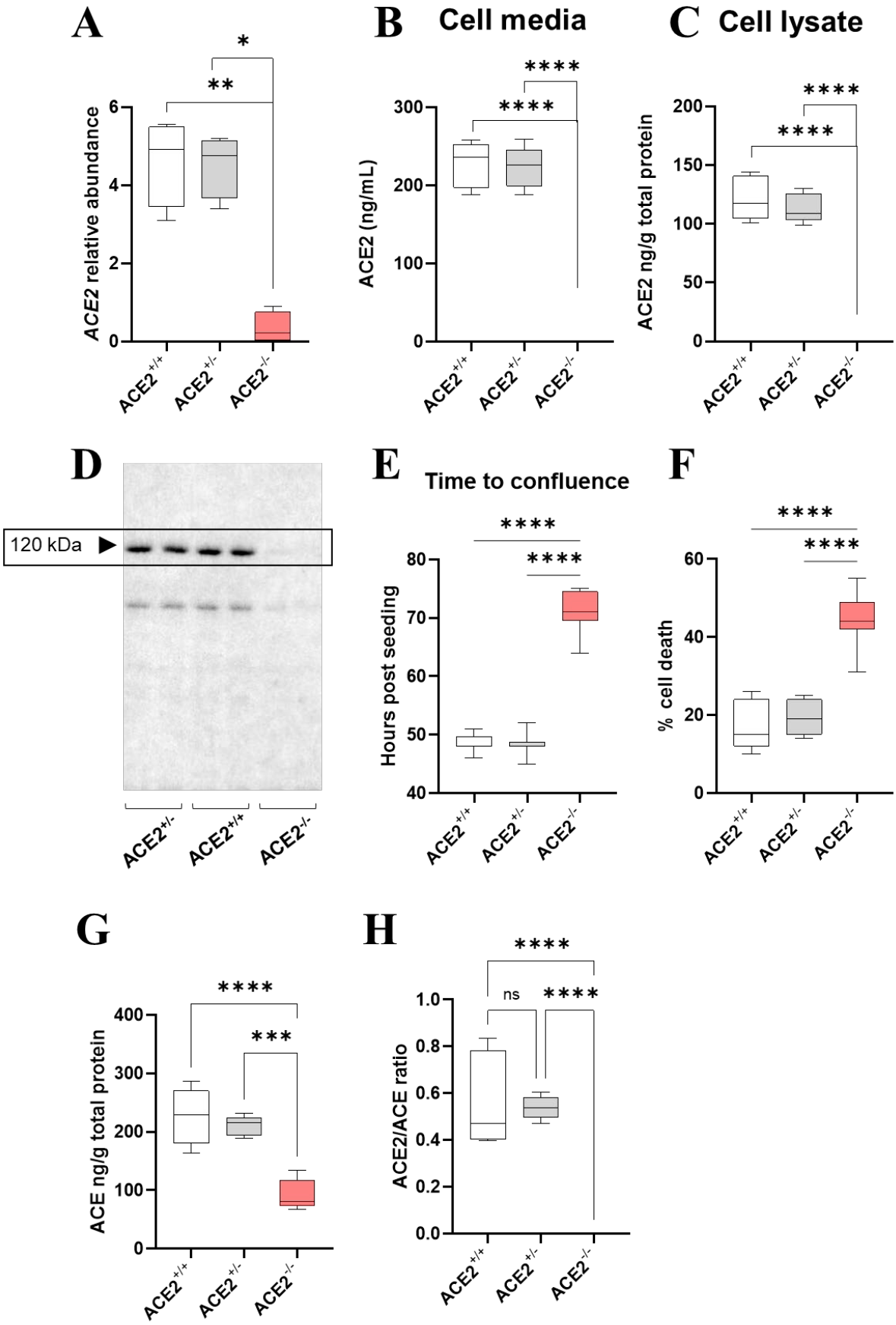

A
ACE2 relative abundance
0
2
4
6
*
**
ACE2+/+
ACE2+/-
ACE2-/-
B
Cell media
ACE2 (ng/mL)
0
100
200
300
****
****
ACE2+/+
ACE2+/-
ACE2-/-
C
Cell lysate
ACE2 ng/g total protein
0
50
100
150
200
****
****
ACE2+/+
ACE2+/-
ACE2-/-
D
120 kDa
ACE2+/-
ACE2+/+
ACE2-/-
E
Time to confluence
Hours post seeding
40
50
60
70
80
****
****
ACE2+/+
ACE2+/-
ACE2-/-
F
% cell death
0
20
40
60
****
****
ACE2+/+
ACE2+/-
ACE2-/-
G
ACE ng/g total protein
0
100
200
300
400
****
***
ACE2+/+
ACE2+/-
ACE2-/-
H
ACE2/ACE ratio
0.0
0.2
0.4
0.6
0.8
1.0
****
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)*** *Representative WB showing ACE2 protein expression in cell lysate of* $ACE^{+/+}$*,* $ACE^{+/-}$ *and ACE2 KO organoids.* ***(E)*** *The time to confluence, in hours post seeding, and* ***(F)*** *percentage cell death in* $ACE^{+/+}$*,* $ACE^{+/-}$ *and ACE2 KO trophoblast stem cells (TSCs).* ***(G)*** *Level of ACE protein in cell lysate and* ***(H)*** *ACE2/ACE (ACE2:ACE) ratio (from cell lysate levels) of* $ACE^{+/+}$*,* $ACE^{+/-}$ *and 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)*

362

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

364 Placental organoids tend to exhibit a spherical structure[17, 18]. However, with the *ACE2* knockout
365 we observed significantly smaller placental organoids with 'squashed', i.e. asymmetrical,
366 structures (**Figures 4A-C**). We have therefore measured the horizontal and vertical diameters
367 of organoids from each genotype to determine the effect of *ACE2* on growth and symmetry.
368
369 $ACE2^{+/+}$ and $ACE2^{+/-}$ organoids had significantly larger diameters compared to *ACE2* KO
370 organoids ($p<0.0001$ for all, **Figure 4D**).
371 Organoids were significantly less symmetrical in *ACE2* KO organoids compared with $ACE2^{+/+}$
372 organoids ($p=0.0186$, **Figure 4E**) only.
373

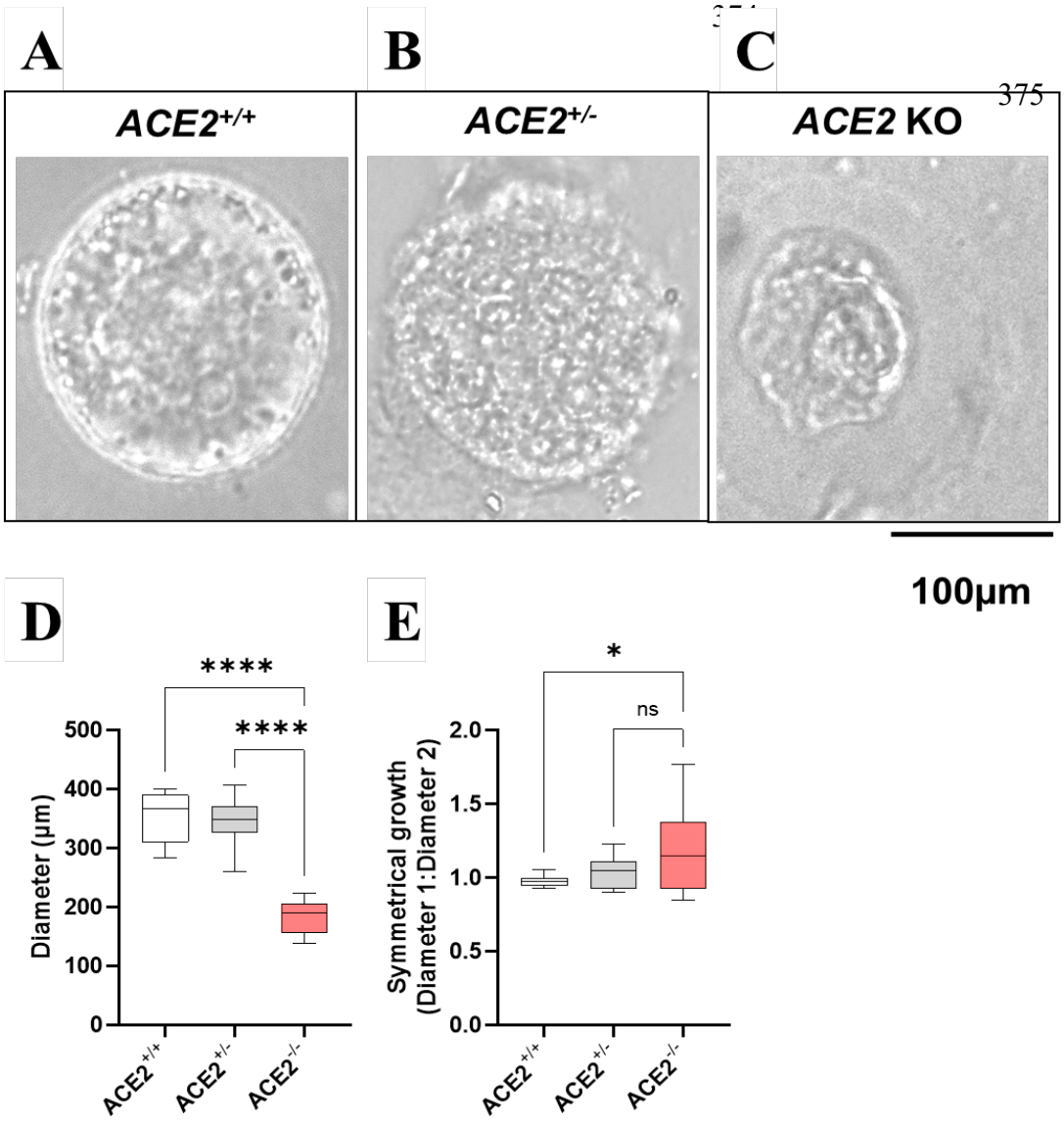


Figure 4. *Representative image (Brightfield) of symmetrical organoid growth (**A**) ACE2$^{+/+}$, (**B**) ACE2$^{+/-}$ and (**C**) asymmetrical organoid growth (ACE2 KO). **(D)** The average diameter (µm) of ACE$^{+/+}$, ACE$^{+/-}$ and ACE2 KO organoids. **(E)** The ratio of diameter 1 (horizontal) 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 5**), 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).

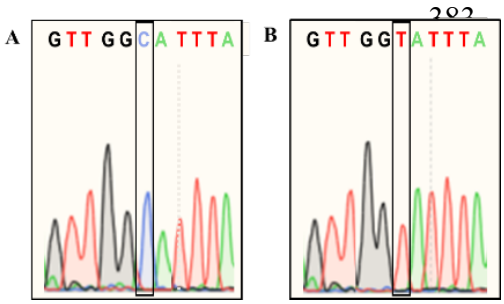


Figure 5. *Successful gene-editing at position 6: the reference C allele (A) and the alternate T allele (B).*

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, 6D**). 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).

399

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

402 There was no significant difference in ACE protein level (measured in cell lysate) between CC,
403 CT and TT organoids (**Figure 6E**). However, the ACE2/ACE ratio was significantly higher in
404 TT organoids compared to both CC and CT organoids (p=0.0049, p=0.0093, respectively;
405 **Figure 6F**). There was no significant difference in ACE protein expression, nor ACE2/ACE
406 ratio, between CC and CT organoids.

407

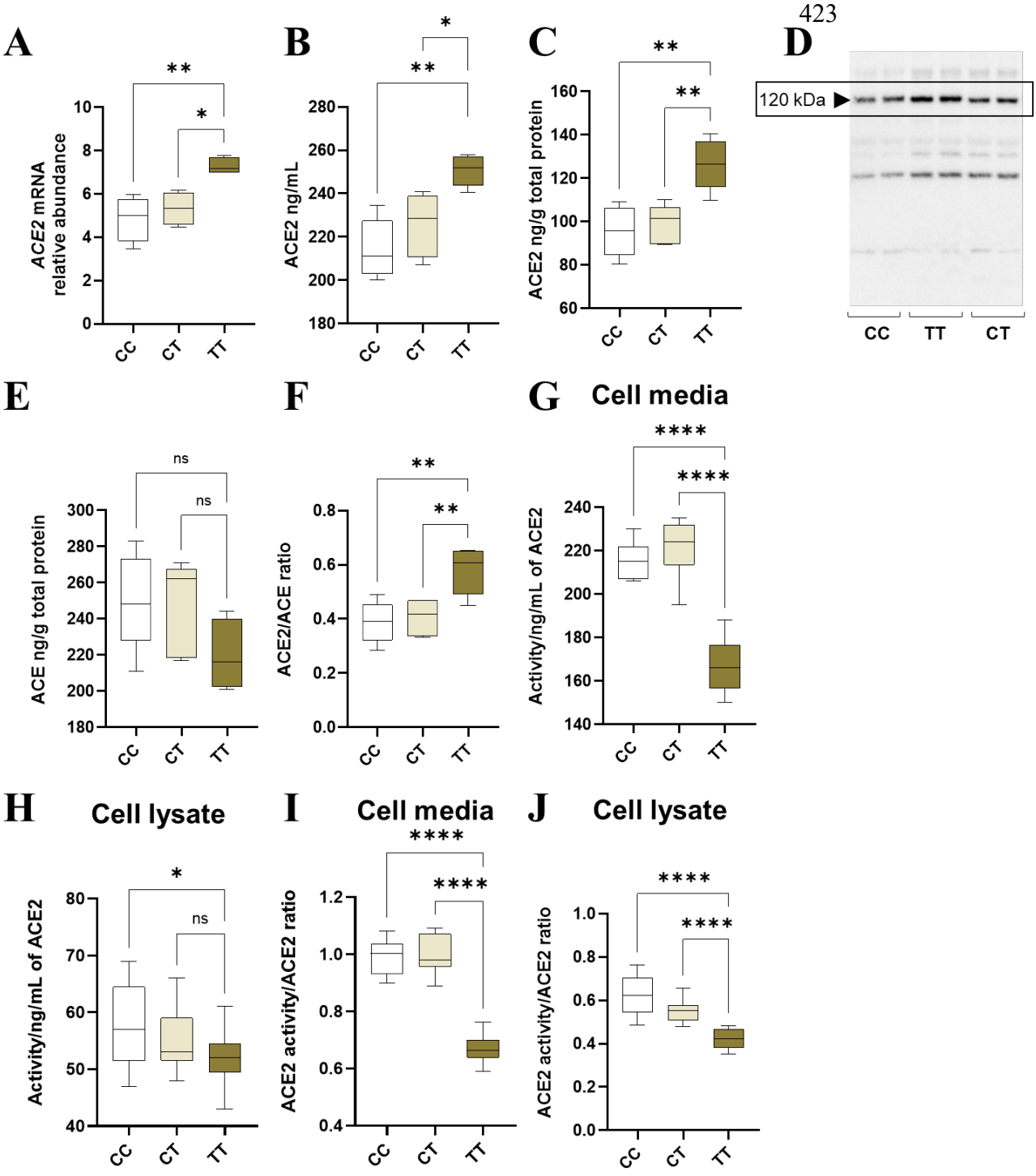
408 ## ACE2 activity is decreased in *ACE2* rs2074192 TT organoids

409 ACE2 enzyme activity was significantly reduced in the cell media of TT organoids compared
410 to both CC and CT organoids (p<0.0001 for both; **Figure 6G**). There was no significant
411 difference in ACE2 activity in the cell media of CT organoids compared to CC organoids.
412 ACE2 enzyme activity was significantly reduced in the cell lysate of TT organoids compared
413 to CC organoids (p=0.0303; **Figure 6H**). There was no significant difference in ACE2 activity
414 in the cell lysate of CT organoids compared to TT organoids, nor compared to CC organoids.

415

416 The ratio between ACE2 activity and ACE2 levels was significantly decreased in the cell media
417 of TT organoids compared to both CC and CT organoids (p<0.0001 for both; **Figure 6I**), while
418 there was no significant difference between CT and CC organoids. The ratio between ACE2
419 activity and ACE2 levels was significantly decreased in the cell lysate of TT organoids
420 compared to both CC and CT organoids (p<0.0001 for both; **Figure 6J**), while there was no
421 significant difference in the ratio between CT and CC organoids.

422


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

424

Figure 6. *The abundance of* ***(A)*** *ACE2 mRNA,* ***(B)*** *ACE2 protein in cell media and* ***(C)*** *ACE2 protein in cell lysate;* ***(D)*** *Representative WB showing ACE2 protein expression in cell lysate of CC, TT and CT organoids.* ***(E)*** *Level of ACE protein in cell lysate,* ***(F)*** *ACE2/ACE ratio (from cell lysate levels); Enzyme activity of ACE2 in* ***(G)*** *cell media and (****H****) cell lysate, and ratio between ACE2 activity and ACE2 levels in (****I****) cell media and (****J****) 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)*

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.

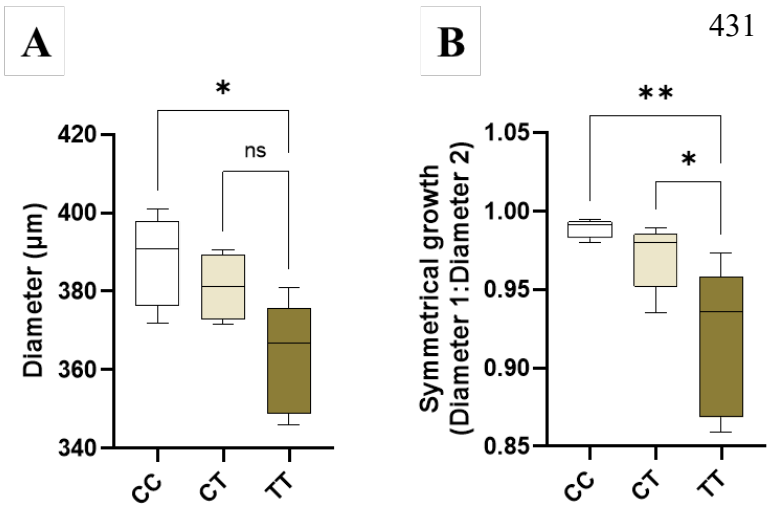


Figure 7. (A) *The average diameter (µm) of CC, CT and TT organoids.* ***(B)*** *The ratio of diameter 1 (horizontal) to diameter 2 (vertical) of CC, CT and TT organoids as a measure of symmetrical organoid growth. A value of 1.0 indicates perfect symmetry. Data are presented as a 10-90 percentile interleaved 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)*

432 ## Discussion

433 This study is the first to successfully use gene-editing to stably produce *ACE2* knockout
434 placental organoids, and the first to induce a SNP in placental organoids. Using these models,
435 the study demonstrates that *ACE2* is important for cell growth (as evidenced by changes in the
436 time to confluence and percentage cell death of TSCs), as well as placental organoid size and
437 growth (as evidenced by changes in organoid diameters and symmetry growth measurements).
438 Furthermore, we show that ACE2:ACE ratio is disrupted in both organoid models, resulting in
439 impaired growth and development. Our data are consistent with our hypothesis that *ACE2* plays
440 an important role in placental development, and that ACE2 and ACE need to exist in a finely
441 tuned balance to ensure proper growth and morphogenesis. We have also confirmed that the
442 *ACE2* rs2074192 TT genotype results in increased ACE2 mRNA and protein, and altered
443 ACE2:ACE ratios, which likely mediate the effects on TSC and organoids growth and cell
444 survival.
445
446 *ACE2* knockout mouse models of pregnancy have been useful in reinforcing the importance of
447 ACE2 in placentation and pregnancy health. This study has extended our understanding of the
448 role of ACE2 in the placenta, creating the first *ACE2* knockout in primary human placenta cells.
449 Furthermore, we have explored the molecular implications of carriage of a common *ACE2*
450 SNP, rs2074192. *ACE2* variants are associated with pregnancy complications, as well as with
451 a number of other diseases characterised by renin-angiotensin system dysfunction, including
452 COVID-19[13,14], hypertension[12, 30] and heart failure[15]. *ACE2* rs2074192 is a common SNP, with
453 an estimated frequency of C (reference allele) = 0.63 and T (alternate allele) = 0.37, according
454 to a study of 102,615 people (including varied ethnicities) in the Genome Aggregation
455 Database[16]. This suggests that at least one copy of this SNP naturally occurs in over one third
456 of the population, and a recent study indicated that the incidence of the TT genotype in their

457 study population was 19.8%[10]. This highlights the importance of understanding the mechanism
458 by which carriage of the alternate allele increases the risk of disease.
459
460 *In silico* analysis of *ACE2* rs2074192 suggests that it increases splicing donor sites, creating a
461 changed secondary RNA structure, affecting protein production and activity. As *ACE2*
462 rs2074192 is an intronic polymorphism, it is likely that it indirectly positively regulates gene
463 expression by altering ACE2 splicing and transcription enhancement[13]. This study confirms
464 that carriage of the TT genotype, but not the CT genotype, results in increased placental ACE2
465 mRNA and protein expression. This has major implications for the pathogenesis of diseases
466 dependent on ACE2 expression, including pregnancy complications such as preeclampsia[1] and
467 SGA[1], and infection with SARS-CoV-2 virus[31], which can directly infect syncytiotrophoblasts
468 through earlier endometrial cell infection[32]. However, the mechanism of action by which this
469 SNP contributes to pathogenesis requires further study. Both the *ACE2* rs2074192 (C to T) SNP
470 and haplotypes CAGC and TAGT are associated with altered levels of the Ang 1-7 peptide, a
471 direct product of ACE2 activity[10]. As this study did not quantify Ang 1-7 expression, further
472 studies should investigate whether Ang 1-7 is altered as a result of *ACE2* rs2074192 genotype,
473 providing additional information on a potential mechanism for pathogenesis of diseases
474 dependent on ACE2 in T allele carriers.
475
476 This study observed that *ACE2* knockout TSCs took longer to reach confluence and had higher
477 percentages of cell death in culture. This finding has implications for pregnancy complications
478 with origins in early gestation, when TSCs play an important role in placental development
479 that underpins subsequent function. During early gestation, the placenta undergoes rapid
480 trophoblast proliferation and differentiation[33]. Not only did this study observe slower TSC
481 growth and higher levels of cell death in *ACE2* knockouts, but *ACE2* knockout organoids had

482 reduced diameters, as well as asymmetrical growth, reflecting impaired organoid development.
483 Interestingly, although no change in TSC growth or cell death was observed with *ACE2*
484 rs2074192 genotype, TT organoids also had smaller diameters and asymmetrical growth.
485
486 This impaired growth has concerning implications for placental development. Human placental
487 organoids provide an innovative model for studying placental development *in vitro*. Ethical
488 limitations to study of early- to mid-gestation placentae, created the need for physiologically
489 relevant human placental models with 3D architecture. Current models are limited[34, 35, 36] while
490 mouse placenta differs both in structure and endocrine function[37]. Organoids recapitulate all
491 first-trimester placental functions, can differentiate into the expected cell lineages, and are able
492 to be cultured long-term without the loss of these features[38]. For this reason, human placental
493 organoids are the closest possible parallel to studying the early gestation human placenta *in*
494 *vivo*. *ACE2* gene-editing of placental organoids in this study provides insight into the
495 detrimental effect of *ACE2* knockout and rs2074192 TT genotype on placental development,
496 likely leading to aberrant placentation in human pregnancy.
497
498 Whilst an absence of ACE2 is clearly detrimental to cell growth and increases cell death, a
499 complete knockout is highly unlikely in nature and thus is less biologically relevant for causal
500 inference. Therefore, we have compared the knockout and induced SNP models to make
501 inferences most biologically relevant to human pregnancy. Surprisingly, both the *ACE2* KO
502 organoids and the induced SNP organoids exhibited diminished growth and symmetry, despite
503 *ACE2* KO organoids having markedly reduced ACE2 protein, and induced SNP organoids
504 having markedly increased protein, compared to their respective controls. This result suggests
505 that the absolute expression value of ACE2 is the not sole determinant of organoid growth.
506

507 One common feature between *ACE2* knockout organoids and TT organoids is the altered
508 ACE2:ACE ratio. Our data suggest that ACE2 and ACE, which are known to partly control cell
509 growth[3], have more contributions to placental organoid development (as evidenced by organoid
510 symmetry and growth) than simply by affecting cell proliferation and death. This is evident
511 from our observation of unchanged TSC growth and cell death levels in TT TSCs, despite them
512 also having altered ACE2 and ACE expression.
513
514 The mechanism for this unexpected increase in ACE2 protein, but decreased organoid growth
515 and symmetry, could be due to the reduced ACE2 enzyme activity observed in TT organoids.
516 *ACE2* rs2074192 is an intronic SNP, which does not directly impact protein coding regions of
517 the genome. Therefore, the contribution of this SNP to ACE2 protein increase is indirect, likely
518 through a complex mechanism not yet elucidated. We hypothesise that the excess of ACE2
519 protein in TT organoids has resulted in a compensatory response in the cell, reducing the
520 *activity* of ACE2 in an attempt to re-establish an ACE2:ACE equilibrium. This theory is
521 supported by the literature, showing that *ACE2* rs2074192 is associated with increased risk of
522 SGA[5], a condition in which inadequate placentation often leads to smaller placentae and a
523 smaller fetus. Furthermore, in SGA, ACE2 activity is significantly reduced[1].
524
525 This study indicates that, whilst ACE2 expression is significant, the activity of the enzyme and
526 maintenance of the ACE2 and ACE in equilibrium (in this paper termed an ACE2:ACE ratio)
527 may be of greater importance. Studies investigating pregnancy complications support this
528 conclusion. In preeclampsia, a potentially life-threatening complication characterised by
529 maternal hypertension and multi-organ failure, ACE2 activity reflected by the ACE2:ACE ratio
530 is higher than normal, impairing placental development and function[1]. In SGA, placental
531 expression of ACE and ACE2 at delivery are both higher compared with uncomplicated

532 pregnancy. Birthweight centiles are negatively correlated with maternal plasma ACE and ACE2
533 levels in mid-pregnancy, suggesting that tight regulation of these enzymes is critical to ensure
534 pregnancy health[1]. As previously mentioned, the ratio of ACE2 activity to ACE2 levels in
535 maternal plasma is significantly reduced in SGA, indicating that ACE2 operates with reduced
536 efficiency in this condition[1]. This could be due to the increased presence of an endogenous
537 inhibitor[39] in SGA, although this has never been investigated. Placental ACE2 deficiency, and
538 the associated elevation in placental Ang II levels, negatively impact pregnancy by impairing
539 maternal weight gain and restricting fetal growth[2]. This implies the balance of ACE2:ACE
540 supersedes the importance of ACE2 levels in isolation in determining fetal growth. Indeed,
541 herein we observed similar reductions in organoid diameter and growth between the complete
542 *ACE2* knockout and rs2074192 models, despite the difference in absolute ACE2 expression
543 values.
544
545 Interestingly, this study showed that *ACE2* rs2074192 TT organoids not only had elevated
546 ACE2 protein within cells (as assessed by cell lysate protein levels) but also secreted more
547 ACE2 protein into the cell media. Whilst ACE2 is typically a membrane-bound peptidase, it
548 can also be shed from the membrane[40], producing soluble ACE2 (sACE2). It should be noted
549 that this study utilised only an ACE2 antibody specific to the membrane-bound ACE2 protein
550 and we cannot comment on the production of sACE2 by these organoid models. Future studies
551 will utilise a specific sACE2 antibody to examine this.
552
553 A limitation of this study is that it only utilised placental tissue from female fetuses. This is
554 because placental expression of ACE and ACE2 is fetal sex dependent. Both ACE and ACE2
555 are more highly expressed in placentae from female compared to male fetuses[41]. For ACE2,
556 this is likely due to the fact that *ACE2*, located on the X-chromosome, escapes X chromosome

557 inactivation, thus often two copies are expressed instead of one in females[42]. The balance of
558 downstream effector peptides Ang II (via ACE) and Ang 1-7 (via ACE2) is also influenced by
559 fetal sex. In early gestation, Ang 1-7:Ang II ratios are reduced in placenta from females,
560 indicating less Ang 1-7 production as a result of reduced ACE2 activity[43]. Female babies are
561 more likely to be growth restricted than males[44, 45]. In this study, the organoid growth symmetry
562 only significantly changed between the *ACE2*$^{+/+}$ and total knockout, but not *ACE2*$^{+/-}$ and the
563 knockout. This could indicate that in males, where only one *ACE2* gene is present (due to the
564 presence of only one X chromosome), there may be differences in placental development. Thus,
565 *ACE2* fetal sex-specific molecular roles require further investigation. Future studies should
566 include male samples, as well as females to expand on the findings from this study.
567
568 Our data clearly demonstrate that ACE2 plays an important role in placental organoid
569 development, and its expression is increased with the rs2074192 TT genotype. Importantly,
570 ACE2 enzyme activity and the ACE2:ACE ratio appeared to make more difference to the
571 growth and development of TSCs and placental organoids than the absolute expression values
572 of ACE2 alone. We anticipate that this study will be used to facilitate a deeper understanding
573 of the role of ACE2 in placental development. The gene-editing technique in placental
574 organoids that we have developed could be used to study the roles of many other genes and
575 their variants on placental growth and function.
576

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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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