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Oxygen and tissue culture affect placental gene expression

O. Brew, M.H.F. Sullivan

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1 **Oxygen and Tissue Culture affect Placental Gene Expression**

2 Brew O[a], Sullivan MHF[b]

3

4 [a] University of West London, Paragon house, Boston Manor Road, Brentford,
5 Middlesex, TW8 9GA

6 [b] Institute of Reproductive & Developmental Biology, Imperial College London,
7 Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN

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10 Abstract

11

12 **Introduction:**

13 Placental explant culture is an important model for studying placental development and
14 functions. We investigated the differences in placental gene expression in response to tissue
15 culture, atmospheric and physiologic oxygen concentrations.

16 **Methods**

17 Placental explants were collected from normal term (38-39 weeks of gestation) placentae
18 with no previous uterine contractile activity. Placental transcriptomic expressions were
19 evaluated with GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix).

20 **Results**
21 We uncovered sub-sets of genes that regulate response to stress, induction of apoptosis
22 programmed cell death, mis-regulation of cell growth, proliferation, cell morphogenesis,
23 tissue viability, and protection from apoptosis in cultured placental explants. We also
24 identified a sub-set of genes with highly unstable pattern of expression after exposure to
25 tissue culture. Tissue culture irrespective of oxygen concentration induced dichotomous
26 increase in significant gene expression and increased enrichment of significant pathways
27 and transcription factor targets (TFTs) including HIF1A. The effect was exacerbated by
28 culture at atmospheric oxygen concentration, where further up-regulation of TFTs including
29 PPARA, CEBPD, HOXA9 and down-regulated TFTs such as JUND/FOS suggest intrinsic
30 heightened key biological and metabolic mechanisms such as glucose use, lipid biosynthesis,
31 protein metabolism; apoptosis, inflammatory responses; and diminished trophoblast
32 proliferation, differentiation, invasion, regeneration, and viability.
33
34 **Discussion**
35 These findings demonstrate that gene expression patterns differ between pre-culture and
36 cultured explants, and the gene expression of explants cultured at atmospheric oxygen
37 concentration favours stressed, pro-inflammatory and increased apoptotic transcriptomic
38 response.

39

40

41 **Keywords**

42 Placenta; Gene Expression; atmospheric oxygen concentration; physiologic oxygen
43 concentration; Tissue Culture

44

45

46 ***Abbreviations***

47 Atmospheric Oxygen Concentration: AOC; Physiologic Oxygen Concentration: POC;
48 AGE: Absolute Gene Expression; RGE: Relative Gene Expression; CHE: Consistent
49 High Expression; CLE: Consistent Low expression

50

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53 commercial, or not-for-profit sectors.

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1 **Introduction:**

2 In a recent publication [1] we reported on the damaging effect of atmospheric oxygen
3 concentration (AOC) on placental explant morphology and RNA quality. We showed
4 that while explants were viable after 6 days culture, there were more syncytial
5 detachment and loss in explants cultured at the AOC (20%) than in physiological
6 oxygen concentration (POC) for term placentae (8% oxygen), and that the RNA quality
7 and integrity of explants cultured at the AOC declined in tandem with
8 syncytiotrophoblast (STB) degeneration, damage and loss. This work confirmed
9 previous reports that while AOC has generally been used during culture, it could be
10 argued that 8% oxygen reflects *in vivo* physiology, and may provide optimal culture
11 conditions for placental villous explants [2-7]. Yet, there has been no previous
12 systematic report on the effects of AOC or POC during explant culture on term placental
13 transcriptomic response to aid interpretations and discrimination between experimental
14 treatment effect and culture oxygen effect. Our objectives therefore, were to investigate
15 the differences in placental transcriptomic changes in response to tissue culture and
16 oxygen, and the genetic alterations and pathways associated with POC and AOC
17 culture.

18

19 **Methods**

20 **Tissue collection and culture**

21 We collected placental explants from 6 placentae with no previous uterine contractile
22 activity following ethics permission (granted by the Hammersmith and Queen

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23 Charlotte's & Chelsea Hospitals Research Ethics Committee) and written informed
24 consent from patients. Details of methods used for placental collection, dissection, and
25 explant culture and viability assessment have been published elsewhere [1]. In brief,
26 approximately 2 cm^3 term (38-39 weeks of gestation) human placental explants were
27 randomly cut (3/placenta) immediately after delivery by elective Caesarean Section from
28 healthy looking areas about 5 cm away from the umbilical cord of normal pregnancies.
29 As previously described [1] micro explants (<50 mg wet weight) of villous tissue were
30 dissected from each sample (3 micro explants/sample) and cultured on 15mm diameter
31 Netwell inserts with 74μm polyester mesh bottoms attached to polystyrene inserts
32 (Corning, UK) and incubated at the liquid-gas interface in POC (8% oxygen, 5% CO_2) or
33 AOC (95% air; 5% CO_2). The micro explants were cultured in RPMI 1640 culture
34 medium (Invitrogen, UK) supplemented with 2 mM L-glutamine, 10% fetal bovine
35 serum, 100 IU/ml penicillin and 100 μg/ml streptomycin for 6 days. The culture media
36 were replaced at days 2 and 4: the medium was placed in a sterile container and
37 exposed to the appropriate oxygen tension for 2 hours before the change of medium
38 was done. The time for the change of medium was kept to the minimum needed (less
39 than 5 minutes). The explants at the end of the culture period were stored immediately
40 in RNAlater (Ambion) at -80℃. Pre-culture, 0 h con trol samples obtained from fresh
41 placentae were stored similarly in RNAlater within 30 minutes of delivery.
42

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45 **RNA and Microarray Preparation**

46 RNA was extracted from explants cultured to time-point 120 h according to
47 Chomczynski method [8] with TRIZOL reagent (Invitrogen, UK) and quality assessed.
48 Details of the methods for RNA extraction, quality and integrity assessments are
49 previously published [1]. Total RNA was processed into labelled cDNA with NuGEN™
50 Ovation™ RNA Amplification System V2 and FL-Ovation™ cDNA Biotin Module V2
51 (Nugen). The resultant fragmented and labelled cDNA was added to the hybridisation
52 cocktail in accordance with the NuGEN™ guidelines for microarray hybridisation onto
53 Affymetrix GeneChip® Human Genome U133 Plus 2.0 arrays (sample per array) in
54 Affymetrix GeneChip® Hybridisation Oven 640 for 18 hours at 45℃. Features that
55 retained bound labelled cRNA after washing were visualized using the GeneChip®
56 Scanner 3000 (Affymetrix). The microarray data was published in the Gene Expression
57 Omnibus (GEO) repository with accession number GEO: GSE74446.

58

59 **Array Quality Control and Processing**

60 Quality control (QC) of the microarray raw data was assessed with Expression Console
61 (Affymetrix) for .CEL files integrity. Probes with unusual signal patterns or signal
62 strength and arrays showing low correlation between hybridization controls thus failed
63 this initial QC measures were excluded from further analysis. The .CEL raw data were
64 imported and processed with Robust Multi-array Average (RMA) into BRB-Array Tools
65 version 4.5.1 – Stable [9], and further processed using the R bionconductor packages
66 including Affy, annotate, annaffy, gcrma, globaltest, GO.db, lumi, ROC, simpleaffy,

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67 bitops, car, gplots, GSA, impute, lars, matlab, pamr, randomForest. The arrays were
68 log2-transformed and quantile-normalised to fit into linear model and a common scale to
69 generate expression measure for each probe set on each array. The spot filter analysis
70 was performed to remove spots whose signals were wrong due to small quantity of
71 cDNA in the array, or errors during the scanning process. Furthermore, genes showing
72 minimal variation across the set of arrays were excluded from the analysis. Genes
73 whose expression differed by at least 1.5 fold from the median in at least 20% of the
74 arrays were retained.

75

76 **Expression pattern analysis**

77 We performed Relative Gene Expression (RGE) analysis for differentially expressed
78 genes between the AOC (experimental) and POC (control) classes using a random-
79 variance t-test. The random-variance t-test permitted sharing of information among
80 genes about within-class variation without assuming that all genes have the same
81 variance [10]. This was supported with a Goeman's global test of whether the
82 expression profiles differed between the classes by multiple permutation of the labels of
83 which arrays corresponded to which classes [11]. We also developed models to identify
84 genes whose expression profiles could predict expression from explants cultured in
85 AOC at the $p < 0.01$ as assessed by the random variance t-test. Compound Covariate
86 Predictor (CCP), Diagonal Linear Discriminant Analysis (DLDA), Nearest Neighbor
87 Classification (NNC), and Support Vector Machines (SVM) with linear kernel [12-15],
88 were used to develop the models. Leave-one-out cross-validation (LOOCV) method
89 was used to compute mis-classification rate. The class labels were randomly permuted

90 (100 random permutations) and the entire LOOCV process was repeated. We further
91 performed Absolute Gene Expression (AGE) analysis [16] using RankProd statistics
92 implemented in MEV_4.9 [17], at FDR < 0.001 (Confidence (1-alpha): 99.9%) to identify
93 within class significant genes that were consistently expressed at high levels or low
94 levels respectively for pre-culture, AOC and POC classes. Genes that were consistently
95 expressed at high (CHE) levels in all the respective phenotype samples were classified
96 as positive significant genes. Alternatively, genes that were consistently expressed at
97 low (CLE) levels in all the respective phenotype samples were classified as negative
98 significant genes. Genes that were not expressed consistently as either high or low in all
99 the samples were classified as non-significant genes.

100

101 **Biological Significance analysis**

102 Gene Ontology (GO), The National Cancer Institute, (Bethesda MD), experimentally
103 verified transcription factor target (TFT) database and Kyoto Encyclopedia of Genes
104 and Genomes (KEGG) and BioCarta biological pathways were evaluated with functional
105 class scoring analysis as described by Pavlidis [18] to identify differential expression of
106 biologically relevant gene-sets between AOC and POC cultured explants. Significant
107 gene-sets for differential expression were summarised with: (i) the Fisher (LS) statistics
108 (provides average log p values for the genes in the target class), (ii) the KS statistics
109 (Kolmogorov-Smirnov statistic computed on the p values for the genes in the target
110 class) and (iii) Gene Set Analysis (GSA) using Maxmean statistics [19] in BRB-array
111 Tools. For comparative analysis of significant gene-set enrichment within pre-culture

112 time zero, POC cultured and AOC explants, the respective gene pools from the AGE
113 analysis were tested with Benjamini-Hochberg statistics [20] implemented in
114 WebGestalt 2013 [21] to identify biologically relevant GOs, TFTs and KEGG biological
115 pathways.

116

117 Results

118 Absolute Gene Expression in Pre-culture and Cultured Explants

119 As a preface to this study, we extensively evaluated RNA extraction and quality control
120 (QC) methods to ensure a high standard of quality for the RNA samples used (results
121 published in [1]). The biological replicate samples were representative of a realistic
122 application of microarrays in placental biology. Thus, following the normalisation and
123 quality control steps, 20,233 genes were used in further analyses. We performed AGE
124 analysis using One Class RankProd statistics to identify 'consistently high expressed'
125 (CHE) and 'consistently low expressed' (CLE) significant genes respectively in Time
126 Zero (pre-culture) explants (designated as T0), and explants cultured in POC (8%) and
127 AOC (20% oxygen for 6 days. FDR Confidence (1-alpha) was set at 99.9% (FDR
128 <0.001).

129 Total of 635, 1207 and 1760 significant genes were consistently expressed in pre-
130 culture (T0); POC and AOC cultured explants respectively (Suppl. Table 1). Of these,
131 224 genes were exclusively expressed in pre-culture samples (Figure 1A, sub-set 2). In
132 contrast, 69 genes (1 CHE and 68 CLE) were exclusively expressed in POC and 574

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133 genes (292 CHE, and 282 CLE) in AOC oxygen cultured explants only (Figures 1 A & B;
134 Suppl. Table 1). We also identified 48 genes that were common to both pre-culture and
135 AOC explants only. Interestingly, CHE genes appeared insensitive to POC, but rather to
136 tissue culture and AOC. All CHE genes (except *SIGLEC6*) in POC explants were also
137 expressed consistently at high levels in AOC explants (Suppl. Table 2). There was a
138 core set of 770 consistently expressed significant genes (Figures 1 A and B sub-sets
139 3&5; Suppl. Tables 2 & 3) that were common to both AOC and POC samples only, and
140 these appear to be genes suggestively responding to tissue culture per se. No genes
141 were exclusively expressed consistently between pre-culture and POC samples only.

142

143 **Relative Gene Expression in Atmospheric and Physiological Oxygen**
144 **Concentration Cultured Explants**

145 We subsequently performed biological relevance network analysis [22] to determine the
146 mutually biological relevance for performing relative gene expression analysis between
147 pre-culture, and explants cultured for 6 days. The results showed no biologically
148 relevant mutual networks between pre-culture and the cultured explants (Suppl. Paper
149 1). We therefore performed the RGE analysis using a two-sample random-variance t-
150 test for differentially expressed genes between the AOC (experimental) and POC
151 (control) oxygen treated classes only and identified 157 significant genes ($p<0.05$). The
152 expression pattern was visualised with a Volcano plot (Figure 1C). We observed 88 up-
153 regulated (Suppl. Table 4) and 69 down-regulated genes (Suppl. Table 5) in AOC
154 relative to POC explants. We further examined whether these genes could be

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155 associated with explant culture in AOC. We thus performed class prediction modelling
156 incorporating Leave-one-out cross-validation and ROC curves. The analysis confirmed
157 the expression of 134 genes as significantly ($p<0.01$) associated with prolonged explant
158 culture at AOC (Suppl. Table 6). Of these, 12 genes were strongly associated ($p<0.001$)
159 with the AOC (Table 1). Three prediction algorithms: CCP, DLDA, and Bayesian
160 compound covariate predictor (BCCP) were used to generate a ROC curve (Figure 1D).
161 The analysis showed a very comparable ROC for all three algorithms with AUC of
162 0.82(CCP), 0.81(DLDA), 0.81(BCCP) (Figure 1D)

163

164 **Ontologies of Genes responding to Physiological and Atmospheric Oxygen**
165 **Concentrations Differ**

166 Table 2 shows a summary comparison of GOs, pathways and TFTs over-represented in
167 the pre-culture and cultured explants gene signatures (detailed in Suppl. Paper 2).
168 Figures 1 A & B sub-sets 3 and 5 contain a preserved set of genes that appears to
169 respond to tissue culture irrespective of oxygen concentration. We therefore examined
170 the GO, pathways and TFT associated with these preserved genes. The sub-set 3
171 genes (CHE genes present in both AOC and POC cultured explants only, irrespective of
172 oxygen concentration) significantly ($p<0.001$) enriched a cluster of cathartic ontologies
173 including programmed cell death, cell death, death, stress response, protein metabolic
174 process, electron transport activity, RNA translation and oxidoreductase activity (Table
175 3; Suppl. Table 10). The sub-set 3 genes also affected regulation of cytosol,
176 cytoplasmic and organelle parts of the placental cells. In contrast, sub-set 5 genes (CLE
177 genes present in both AOC and POC explants only irrespective of oxygen

178 concentration) were mostly associated with the GO terms related to cellular
179 communication including multicellular organismal signalling, receptor binding, ionotropic
180 receptor, 3',5'-cyclic-GMP phosphodiesterase activity and ionotropic glutamate receptor
181 complex (Suppl. Table 11).

182

183 In addition, the AOC only CLE genes (Figure 1B sub-set 8) significantly enriched
184 specific GO terms including Passive transmembrane transporter activity, ion gated
185 channel activity, calmodulin-dependent cyclic-nucleote phosphodiesterase activity, and
186 cation channel activity (Suppl. Table 12), while the CHE genes (Figure 1A sub-set 4)
187 further enriched proteolysis involved in cellular protein catabolic process, ubiquitin-
188 dependent protein catabolic process, ligase activity, threonine-type peptase activity and
189 ubiquitin-protein ligase activity (Suppl. Table 13). There was no specific up-regulated
190 gene set for the POC explants. However, the POC only CLE genes (Figure 1B sub-set
191 7) significantly enriched specific molecular functions involved in multicellular organismal
192 process and voltage-gated ion channel activity (Suppl. Table 14).

193

194 **Effects of Atmospheric relative to Physiological Oxygen Concentrations**
195 We further examined closely the effects of AOC relative to POC on placental
196 transcription factor target genes and biological pathway gene-sets. We used a two-
197 sample random variance T-test design, LS/KS permutation test and Efron-Tibshirani's
198 GSA maxmean to probe the National Cancer Institute, (Bethesda MD), experimentally
199 verified transcription factor target database. A total of 73 TFT gene-sets were

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200 investigated and 15 (6 up-regulated and 9 down-regulated in AOC relative to POC)
201 were significantly ($p<0.05$) enriched (Table 4). The down-regulated TFT gene sets
202 including REL, ETV4, ATF3, STAT1, JUND and STAT5B are seemingly involved in cell
203 growth, proliferation, invasion, regeneration, differentiation, transformation, tissue
204 viability, protection from apoptosis, and glands development (Table 4). Conversely, the
205 up-regulated TFTs such as HIF1A, PPARA, CEBPD, STAT3, and CEBPE are mostly
206 associated with oxygen regulation, immune and inflammation responses; leptin
207 mediated response; lipid metabolism; suppression and mis-regulation of cell growth and
208 proliferation; cell morphogenesis; induction of apoptosis; and oxygen regulation (Table
209 4).
210
211 Results from the RGE pathway analyses were consistent with the AGE GO and
212 transcription factor target gene sets analysis. For example, as expected Peroxisome,
213 Nuclear Receptors in Lipid Metabolism and Toxicity, Glycerolipid metabolism, and
214 Reversal of Insulin Resistance by Leptin pathways were up-regulated (Tables 5; Suppl.
215 Tables 15 and 16) in line with PPARA and STAT3 TFT gene-sets up-regulation.
216 Similarly, RNA degradation, Nucleotide excision repair, and mRNA surveillance
217 pathway were up-regulated in line with over-expression of CEBPE target gene-set.
218
219
220
221 **Discussion**

222 It is well accepted that placental explant culture is an important model for studying
223 placental transport, proliferation, differentiation, morphology, metabolism and endocrine
224 functions under conditions akin to normal physiology. It is also clear that gestational age
225 mismatch with *ex vivo* oxygen concentration affects these placental functional
226 properties [23,24]. In this report, we have provided comprehensive evidence on
227 differences in transcriptomic expression underpinning general cell biology and
228 biochemical processes in response to placental explants culture at AOC and POC. A
229 key finding is the observation of the dichotomous increase in the number of significant
230 placental genes consistently expressed in line with oxygen concentration. Whereby,
231 more genes were consistently expressed at high levels than were expressed at low
232 levels. Interestingly, the disparate transcriptomic response not only affected parts or
233 extracellular environment of the placenta cells but also impacted on the elemental
234 activities of the gene products at the molecular level, such as binding or catalysis.
235
236 The uncovering of the placental gene sub-sets mediating programmed cell death;
237 response to stress; cell differentiation, and inhibition of cell proliferation was novel.
238 Albeit transcriptomic pathways at the moment, the findings are nonetheless exciting as
239 the current data provides evidence to encourage review of our understanding and
240 further study of the effects of culture conditions on trophoblast apoptosis, differentiation
241 and proliferation. For, it is reported previously that trimester 1 villous explant culture in
242 approximately 3% oxygen stimulates increased trophoblast proliferation, while culture in
243 AOC appears to support proliferation but prevents invasiveness; and that AOC
244 increases apoptosis in term villous explants [25-27].

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245

246 Our current findings introgressively suggest that placental explant response to tissue
247 culture per se induces expression of genes with preponderance towards apoptosis
248 irrespective of culture oxygen concentration. And that, the magnitude of the
249 programmed cell death could be sensitive to AOC. For example, we observed that AOC
250 was associated with down-regulation of trascription factor JUND target gene set
251 involved in apoptosis protection. Moreso, we observed relative up-regulation of PPARA
252 TFT gene set in explants cultured at AOC. Thus, considering that PPARA ligand
253 activation has been linked to induction of apoptosis [28,29], it is plausible to surmise
254 that AOC could exacerbate placental programmed cell death through activation of the
255 pro-apoptotic target gene sets via PPARA TFT. Furthermore, PPARA is associated with
256 lipid metabolism, cell differentiation, inhibition of cell proliferation, up-regulation of
257 immune and inflammation responses [26,27]. Therefore, it is equally consistent to
258 extend the suggestion that poor trophoblast proliferation associated with AOC could be
259 mediated through activation of PPARA TFT gene set.

260

261 Certainly, HIF1A is well known to be constitutively expressed in the placenta to mediate
262 hypoxic adaptation during placentation [30]. It has also been suggested that HIF-1A can
263 be induced by factors other than hypoxia, including placental hormones, cytokines and
264 growth factors, and well-oxygenated environment (~20% oxygen) [23,30,31]. We
265 observed that HIF1A TFT gene sets were significantly enriched in response to AOC.
266 The comparative analysis also showed that while no HIF1A target gene set was
267 significantly enriched in pre-culture explants, the homolog V$HIF1_Q3 containing the

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268 motif GNNKACGTGCGGNN target gene set was significantly enriched in both AOC and
269 POC samples. In addition, the target gene set for V$HIF1_Q5 containing the motif
270 CGTACGTGCNGB was also enriched in explants cultured in AOC. Thus, suggesting
271 that high oxygen could be an intrinsic regulatory mechanism for HIF activation in human
272 placenta.
273
274 Our findings do not only provide evidence to confirm that well oxygenated (non-hypoxic)
275 environment regulates HIF1A target gene sets in the placenta, but also provide exciting
276 insights into the regulation of placental development through activator protein-1 (AP-1;
277 FOS/JUND). AP-1 proto-oncogenes have been linked to regulation of placental gene
278 expression in relation to oxygen concentration [32,33]. Our finding showed that JUND
279 and FOS target gene sets were also significantly enriched in response to AOC. While
280 our current data does not provide evidence on the interplay between HIF1A and AP1
281 pathways in the placenta, there are previous reports that suggest AP-1 may synergise
282 with HIF-1 to regulate hypoxic gene expression in the placenta [32-36]. It is therefore
283 plausible to suggest for further work that HIF1 and AP-1 target gene sets could provide
284 a co-regulatory response or feedback pathways in the regulation of placental
285 development.
286
287 Indeed, we have provided novel transcriptomic evidence not only to show that explant
288 culture per se could trigger placental gene sets that regulate programmed cell death
289 and stress response but also to support the consensus that AOC is pathological for
290 placental explant culture [6] by exacerbating a primed defective response.

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395 Tables

396 **Table 1: Genes significantly associated with 20% oxygen culture**

Symbol	Name	Fold-change	t-value	p-value
GNAZ	guanine nucleotide binding protein (G protein), alpha z polypeptide	-1.95	-5.999	0.000103
NPPB	natriuretic peptide precursor B	-3.37	-5.917	0.000115
AIG1	androgen-induced 1	-1.46	-5.468	0.000221
C10orf90	chromosome 10 open reading frame 90	-1.95	-5.003	0.000444
TPH1	tryptophan hydroxylase 1	-1.69	-4.873	0.000543
PNMA2	paraneoplastic antigen MA2	-2.55	-4.855	0.000558
PRPS1	phosphoribosyl pyrophosphate synthetase 1	-1.85	-4.782	0.000626
MMP12	matrix metallopeptidase 12 (macrophage elastase)	-2.67	-4.546	0.00091
NEU1	sialidase 1 (lysosomal sialidase)	1.29	4.529	0.000936
C10orf12	chromosome 10 open reading frame 12	1.7	4.566	0.000882
LOC100129890	similar to hCG1750329	1.58	5.029	0.000427
ABHD4	abhydrolase domain containing 4	1.57	6.331	0.0000651

397 The prediction rule was defined by the inner sum of the weights (w_i) and log intensity expression (x_i) of significant genes.
398 Modelled predictors: A sample was classified to the class **20% oxygen** if the sum was greater than the threshold; that is,
399 $\sum_i w_i x_i >$ threshold. The threshold for the Compound Covariate predictor = -155.831; threshold for the Diagonal Linear
400 Discriminant predictor = 318.343; threshold for the Support Vector Machine predictor = -7.845 (supplementary Table 4 contains
401 full list of 20% oxygen concentration culture genes).

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Table 2: Enrichment analysis summary table

Enrichment	Biological Processes	Molecular Process	Cellular Component	KEGG Pathways	Transcription Factor Targets
Con Total	40	11	40	42	251
8% Total	40	15	40	61	400
20% Total	40	23	40	121	497
Overlap					
Enriched in Pre-culture only	5	2	9	2	4
Enriched in 8% Oxygen only	2	1	4	0	7
Enriched in 20% Oxygen only	1	8	3	55	92
Enriched in all explants	32	8	30	35	235
Enriched in Pre-culture and 8% only	1	0	0	0	0
Enriched in Pre-culture and 20% only	2	1	1	5	12
Enriched in 8% and 20% only	5	6	6	26	158

Details of enriched pathways and GO terms are provided in supplementary tables

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Table 3: Ontologies of Genes Responding to Tissue Culture Irrespective of Oxygen Concentration

Database	GO ID	Name	C	O	E	R	rawP	adjP
BP	GO:0032268	regulation of cellular protein metabolic process	1250	64	27.85	2.3	1.89E-10	3.89E-07
BP	GO:0051246	regulation of protein metabolic process	1413	68	31.49	2.16	6.52E-10	4.47E-07
BP	GO:0006091	generation of precursor metabolites and energy	451	34	10.05	3.38	5.03E-10	4.47E-07
BP	GO:0006950	response to stress	2952	111	65.78	1.69	2.29E-09	1.18E-06
BP	GO:0016265	death	1706	74	38.01	1.95	9.60E-09	2.82E-06
BP	GO:0008219	cell death	1704	74	37.97	1.95	9.13E-09	2.82E-06
BP	GO:0009987	cellular process	12899	316	287.43	1.1	9.01E-09	2.82E-06
BP	GO:0022900	electron transport chain	148	17	3.3	5.15	3.28E-08	8.04E-06
BP	GO:0016071	mRNA metabolic process	613	37	13.66	2.71	3.52E-08	8.04E-06
BP	GO:0012501	programmed cell death	1545	67	34.43	1.95	5.90E-08	1.21E-05
MF	GO:0005515	protein binding	7337	215	155.05	1.39	8.23E-12	2.83E-09
MF	GO:0003723	RNA binding	854	41	18.05	2.27	7.36E-07	0.0001
MF	GO:0008092	cytoskeletal protein binding	638	32	13.48	2.37	5.46E-06	0.0006
MF	GO:0003743	translation initiation factor activity	50	8	1.06	7.57	9.04E-06	0.0006
MF	GO:0015078	hydrogen ion transmembrane transporter activity	101	11	2.13	5.15	9.26E-06	0.0006
MF	GO:0004129	cytochrome-c oxase activity	28	6	0.59	10.14	2.16E-05	0.0009
MF	GO:0016676	oxoreductase activity, acting on a heme group of donors, oxygen as acceptor	28	6	0.59	10.14	2.16E-05	0.0009
MF	GO:0015002	heme-copper terminal oxase activity	28	6	0.59	10.14	2.16E-05	0.0009
MF	GO:0016675	oxoreductase activity, acting on a heme group of donors	29	6	0.61	9.79	2.68E-05	0.001
MF	GO:0005488	binding	11955	281	252.65	1.11	3.14E-05	0.0011
CC	GO:0005737	cytoplasm	9130	267	185.24	1.44	3.32E-20	8.80E-18
CC	GO:0044444	cytoplasmic part	6772	208	137.4	1.51	8.35E-15	1.11E-12
CC	GO:0005829	cytosol	2372	101	48.13	2.1	7.20E-14	6.36E-12
CC	GO:0044424	intracellular part	12237	304	248.28	1.22	2.98E-13	1.97E-11
CC	GO:0005622	intracellular	12564	306	254.92	1.2	7.36E-12	3.90E-10
CC	GO:0043226	organelle	10651	268	216.11	1.24	1.17E-09	5.17E-08
CC	GO:0043229	intracellular organelle	10636	267	215.8	1.24	2.00E-09	7.57E-08
CC	GO:0044446	intracellular organelle part	6725	189	136.45	1.39	5.69E-09	1.88E-07
CC	GO:0044422	organelle part	6812	190	138.21	1.37	9.62E-09	2.83E-07
CC	GO:0044464	cell part	14643	329	297.1	1.11	1.87E-08	4.58E-07

C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category; E: the expected number in the category; R: ratio of enrichment; rawP: p value from hypergeometric test; adjP: p value adjusted by the multiple test adjustment

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Table 4: Enriched Transcription Factor Target Gene Sets at high oxygen

Transcription Factor Gene-Sets	Function	Number of genes	p-value	Maxmean Di
REL_T00168	A proto-oncogene. Involved in NF-κB transcription. Promotes B-cell survival and proliferation and lymphoma	23	0.00101	(-)
RELA_T00594	Involved in NF-κB dependant cellular metabolism, chemotaxis. Modulates immune responses. Positively associated with cancer.	81	0.00417	(-)
SPI1_T02068	Activates gene expression during myeloid and B-lymphoid cell development. Regulates purine-rich sequence and alternative splicing of target genes.	84	0.00494	(-)
HOXA9_T01709	Regulates gene expression, cell morphogenesis, cell differentiation	9	0.005	(+)
FOS_T00123	AP1 transcription factor complex. Regulates cell proliferation, differentiation, and transformation. Associated with apoptotic cell death.	39	0.005	(-)
HIF1A_T01609	Hypoxia regulation	72	0.01	(+)
ETV4_T00685	Activates matrix metalloproteinase genes. Associated with invasion and metastasis of tumour cell	71	0.01188	(-)
CEBPE_T04883	Transcriptional mis-regulation in cancer.	7	0.01483	(+)
PPARA_T05221	Lipid metabolism, cell differentiation, inhibits cell proliferation. Ups immune and inflammation responses. Induces apoptosis	50	0.01561	(+)
ATF3_T01313	Induced upon physiological stress in various tissues. A marker of regeneration following injury.	11	0.02371	(-)
STAT1_T01492	Mediates and upregulates genes expression for cell viability. Induces cellular antiviral state.	48	0.0256	(-)
CEBPD_T00583	Growth suppression	21	0.02734	(+)
JUND_T01978	AP1 transcription factor complex. Protects cells from p53-dependent senescence and apoptosis.	15	0.03	(-)
STAT5B_T05736	Mediates signal transduction from cytokines and growth hormones. Involved in TCR signalling, apoptosis, mammary gland development.	23	0.03393	(-)
STAT3_T05694	Mediates responses to interleukins, KITLG/SCF, LEP and other growth factors. Cell cycle regulation.	50	0.03948	(+)

Table shows 15 out of 73 investigated gene sets. LS/KS permutation test found 12 significant gene sets. Efron-Tibshirani's maxmean test found 6 significant gene sets (under 200 permutations). (+) and (-) represent respectively, up or down-regulated transcription factor target gene set in high oxygen explants (20%) relative to low oxygen (8%), as determined with Efron-Tibshirani's maxmean test (Detailed of gene-sets in suppl. Table)

Table 5: Significant Pathway Gene Sets Associated with high Oxygen

Pathway	Pathway ID	Pathway description	Number of genes	p-value	Maxmean
KEGG	hsa00030	Pentose phosphate pathway	28	0.00013	(+)
	hsa00561	Glycerolipid metabolism	51	0.00058	(+)
	hsa03420	Nucleotide excision repair	45	0.005	(+)
	hsa04710	Circadian rhythm	23	0.005	(+)
	hsa00310	Lysine degradation	44	0.00665	(+)
	hsa00400	Phenylalanine, tyrosine and tryptophan biosynthesis	5	0.00749	(+)
	hsa00620	Pyruvate metabolism	40	0.00913	(+)
	hsa00053	Ascorbate and aldarate metabolism	18	0.0111	(+)
	hsa04146	Peroxisome	81	0.015	(+)
	hsa03010	Ribosome	87	0.015	(+)
BioCarta	h_vitCBPathway	Vitamin C in the Brain	11	0.005	(+)
	h_ace2Pathway	Angiotensin-converting enzyme 2 regulates heart function	13	0.005	(+)
	h_leptinPathway	Reversal of Insulin Resistance by Leptin	11	0.005	(+)
	h_npp1Pathway	Regulators of Bone Mineralization	10	0.005	(+)
	h_plateletAppPathway	Platelet Amyloid Precursor Protein Pathway	14	0.01	(+)
	h_cardiacegfPathway	Role of EGF Receptor Transactivation by GPCRs in Cardiac Hypertrophy	18	0.01	(+)
	h_erkPathway	Erk1/Erk2 Mapk Signaling pathway	28	0.01354	(+)
	h_alkPathway	ALK in cardiac myocytes	37	0.01593	(+)
	h_nuclearRsPathway	Nuclear Receptors in Lipid Metabolism and Toxicity	35	0.03298	(+)
	h_akap95Pathway	AKAP95 role in mitosis and chromosome dynamics	12	0.035	(+)
KEGG	hsa05150	Staphylococcus aureus infection	53	0.00022	(-)
	hsa05323	Rheumatoid arthritis	88	0.00043	(-)
	hsa00590	Arachidonic acid metabolism	57	0.00379	(-)
	hsa04350	TGF-beta signaling pathway	84	0.00404	(-)
	hsa04610	Complement and coagulation cascades	71	0.005	(-)
	hsa00603	Glycosphingolipid biosynthesis - globo series	14	0.005	(-)
	hsa05014	Amyotrophic lateral sclerosis (ALS)	55	0.005	(-)
	hsa00232	Caffeine metabolism	8	0.00758	(-)
	hsa04940	Type I diabetes mellitus	44	0.01932	(-)
	hsa05144	Malaria	53	0.01969	(-)
BioCarta	h_compPathway	Complement Pathway	19	0.0001	(-)
	h_classicPathway	Classical Complement Pathway	14	0.0006	(-)
	h_antisensePathway	RNA polymerase III transcription	5	0.005	(-)
	h_mspPathway	Msp/Ron Receptor Signaling Pathway	7	0.00708	(-)
	h_eicosanoidPathway	Eicosanoid Metabolism	22	0.00967	(-)

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h_p38mapkPathway	p38 MAPK Signaling Pathway	36	0.01104	(-)
h_lectinPathway	Lectin Induced Complement Pathway	12	0.01205	(-)
h_il10Pathway	IL-10 Anti-inflammatory Signaling Pathway	13	0.01312	(-)
h_alternativePathway	Alternative Complement Pathway	9	0.01335	(-)
h_inflamPathway	Cytokines and Inflammatory Response	29	0.01494	(-)

Table contains the top 10 most significant up and down-regulated biological pathways from KEGG and BioCarta. Supplementary Tables 15 and 16 contain full list of the significantly enriched pathways associated with high oxygen. (+) and (-) represent respectively, up or down-regulated transcription factor target gene set determined with Efron-Tibshirani's maxmean test

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Figure 1: Expressed Genes in Explants Cultured at Physiologic and Atmospheric Oxygen Concentrations

Figure 1A shows overlap of consistent high level expressed (CHE) genes between pre-culture, and explants culture at AOC and POC. Figure 1B shows overlap between pre-culture CHE genes and cultured explants consistent low expressed (CLE) genes. Figure 1C shows a volcano plot of 157 significant genes (blue dots). Random variance model parameters at a= 1.31652, b= 17.03489, Kolmogorov-Smirnov statistic= 0.01 and a nominal significance level (dotted line) of each univariate test at $p < 0.05$ (210 exact permutations). Figure 1D shows the ROC curve from the Bayesian Compound Covariate Predictor for AOC associated genes. S1 – S8 = Gene Sub-sets 1 – 8
T0 = Pre-culture CHE genes; AOC = Atmospheric Oxygen Concentration; POC = Physiologic Oxygen Concentration; +ve = CHE; -ve = CLE.

NB: Print figure 1 in colour

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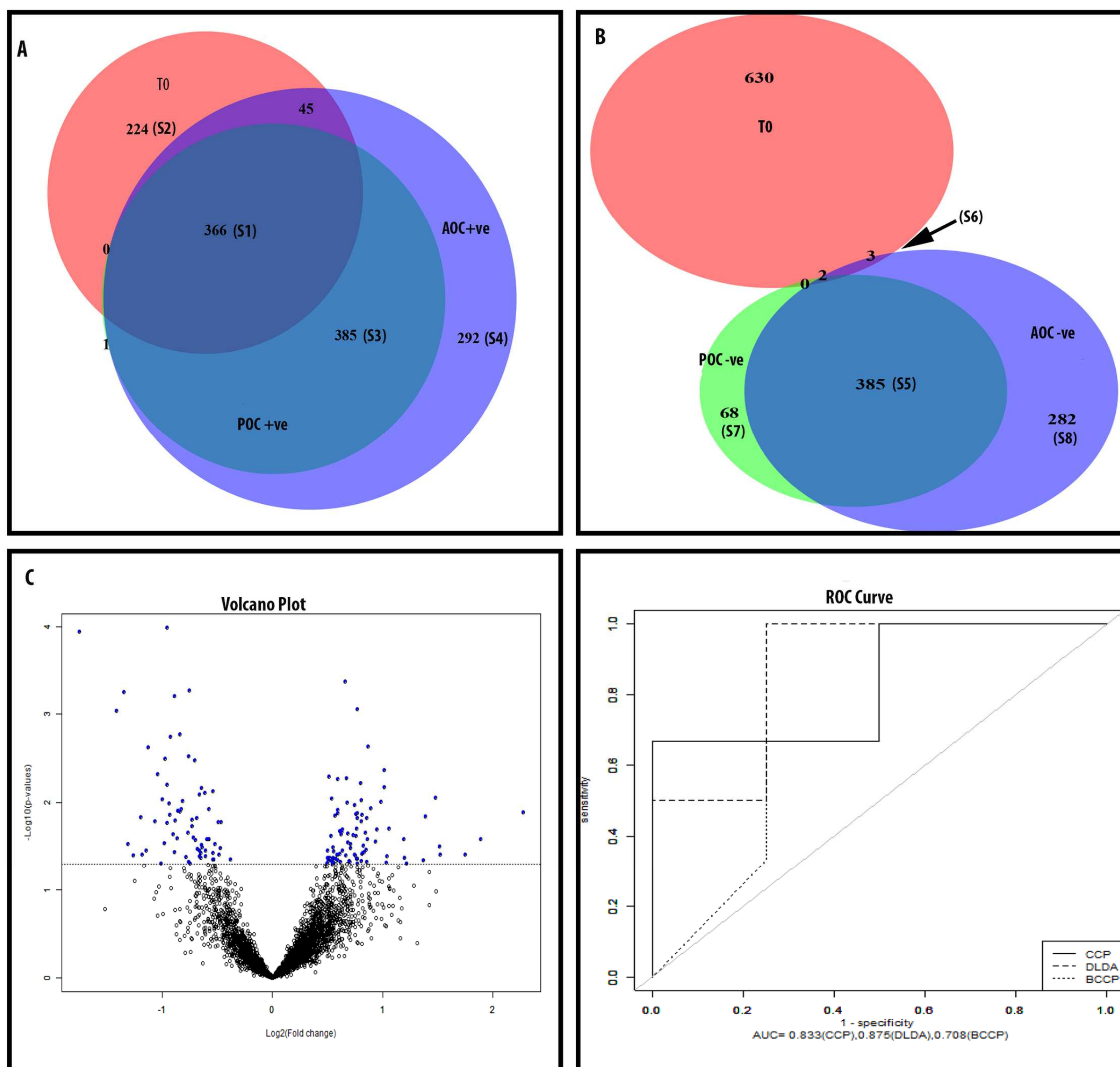

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Highlights: Oxygen and Tissue Culture affects Placental Gene Expression

- Gene expression patterns differ between pre-culture and cultured explants
- Tissue culture up-regulates apoptosis and response to stress genes in placenta
- Atmospheric Oxygen Concentration up-regulates HIF1A transcription target gene set
- Atmospheric Oxygen Concentration regulated genes favour apoptosis and inflammation

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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