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Accepted Manuscript

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PII: S0143-4004(17)30255-2

DOI: 10.1016/j.placenta.2017.04.024

Reference: YPLAC 3642

To appear in: *Placenta*

Received Date: 28 February 2017

Revised Date: 25 April 2017

Accepted Date: 27 April 2017

Please cite this article as: Brew O, Sullivan MHF, Oxygen and tissue culture affect placental gene expression, *Placenta* (2017), doi: 10.1016/j.placenta.2017.04.024.

Oxygen and Tissue Culture affect Placental Gene Expression

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Abstract

Introduction:

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

Methods

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

Results

We uncovered sub-sets of genes that regulate response to stress, induction of apoptosis programmed cell death, mis-regulation of cell growth, proliferation, cell morphogenesis, tissue viability, and protection from apoptosis in cultured placental explants. We also identified a sub-set of genes with highly unstable pattern of expression after exposure to tissue culture. Tissue culture irrespective of oxygen concentration induced dichotomous increase in significant gene expression and increased enrichment of significant pathways and transcription factor targets (TFTs) including HIF1A. The effect was exacerbated by culture at atmospheric oxygen concentration, where further up-regulation of TFTs including PPARA, CEBPD, HOXA9 and down-regulated TFTs such as JUND/FOS suggest intrinsic heightened key biological and metabolic mechanisms such as glucose use, lipid biosynthesis, protein metabolism; apoptosis, inflammatory responses; and diminished trophoblast proliferation, differentiation, invasion, regeneration, and viability.

Discussion

These findings demonstrate that gene expression patterns differ between pre-culture and cultured explants, and the gene expression of explants cultured at atmospheric oxygen concentration favours stressed, pro-inflammatory and increased apoptotic transcriptomic response.

Keywords

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

Abbreviations

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

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Introduction:

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

Methods

Tissue collection and culture

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

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

RNA and Microarray Preparation

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

Array Quality Control and Processing

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

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

Expression pattern analysis

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

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

Biological Significance analysis

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

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

Results

Absolute Gene Expression in Pre-culture and Cultured Explants

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

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

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

Relative Gene Expression in Atmospheric and Physiological Oxygen Concentration Cultured Explants

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

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

Ontologies of Genes responding to Physiological and Atmospheric Oxygen Concentrations Differ

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

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

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

Effects of Atmospheric relative to Physiological Oxygen Concentrations

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

investigated and 15 (6 up-regulated and 9 down-regulated in AOC relative to POC) were significantly ($p<0.05$) enriched (Table 4). The down-regulated TFT gene sets including REL, ETV4, ATF3, STAT1, JUND and STAT5B are seemingly involved in cell growth, proliferation, invasion, regeneration, differentiation, transformation, tissue viability, protection from apoptosis, and glands development (Table 4). Conversely, the up-regulated TFTs such as HIF1A, PPARA, CEBPD, STAT3, and CEBPE are mostly associated with oxygen regulation, immune and inflammation responses; leptin mediated response; lipid metabolism; suppression and mis-regulation of cell growth and proliferation; cell morphogenesis; induction of apoptosis; and oxygen regulation (Table 4).

Results from the RGE pathway analyses were consistent with the AGE GO and transcription factor target gene sets analysis. For example, as expected Peroxisome, Nuclear Receptors in Lipid Metabolism and Toxicity, Glycerolipid metabolism, and Reversal of Insulin Resistance by Leptin pathways were up-regulated (Tables 5; Suppl. Tables 15 and 16) in line with PPARA and STAT3 TFT gene-sets up-regulation. Similarly, RNA degradation, Nucleotide excision repair, and mRNA surveillance pathway were up-regulated in line with over-expression of CEBPE target gene-set.

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].

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

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

motif GNNKACGTGCGGNN target gene set was significantly enriched in both AOC and POC samples. In addition, the target gene set for V$HIF1_Q5 containing the motif CGTACGTGCNGB was also enriched in explants cultured in AOC. Thus, suggesting that high oxygen could be an intrinsic regulatory mechanism for HIF activation in human placenta.

Our findings do not only provide evidence to confirm that well oxygenated (non-hypoxic) environment regulates HIF1A target gene sets in the placenta, but also provide exciting insights into the regulation of placental development through activator protein-1 (AP-1; FOS/JUND). AP-1 proto-oncogenes have been linked to regulation of placental gene expression in relation to oxygen concentration [32,33]. Our finding showed that JUND and FOS target gene sets were also significantly enriched in response to AOC. While our current data does not provide evidence on the interplay between HIF1A and AP1 pathways in the placenta, there are previous reports that suggest AP-1 may synergise with HIF-1 to regulate hypoxic gene expression in the placenta [32-36]. It is therefore plausible to suggest for further work that HIF1 and AP-1 target gene sets could provide a co-regulatory response or feedback pathways in the regulation of placental development.

Indeed, we have provided novel transcriptomic evidence not only to show that explant culture per se could trigger placental gene sets that regulate programmed cell death and stress response but also to support the consensus that AOC is pathological for placental explant culture [6] by exacerbating a primed defective response.

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Tables

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

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

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

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

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	(-)

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

Oxygen and Tissue Culture affect Placental Gene Expression

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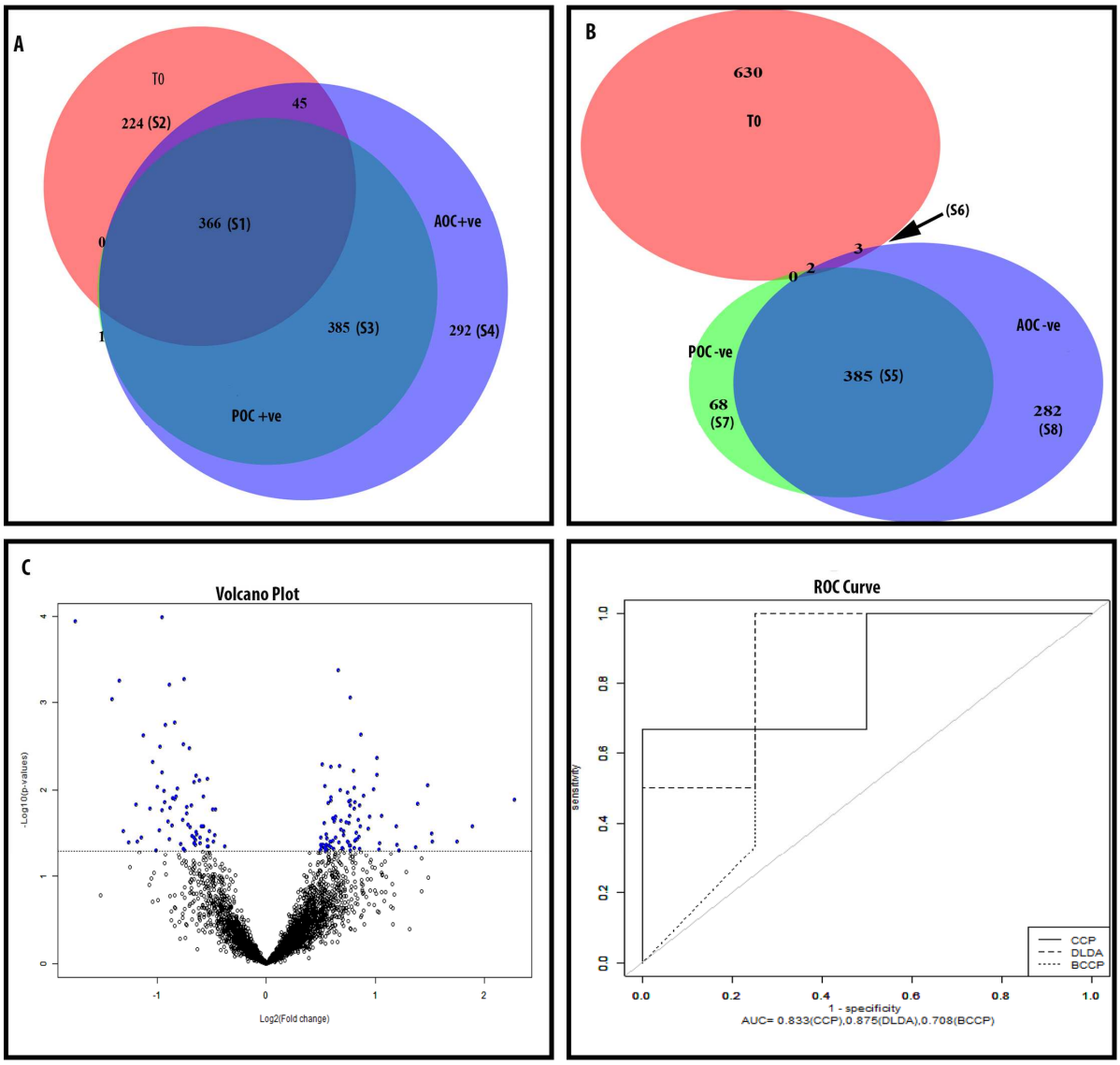
Figure 1: Expressed Genes in Explants Cultured at Physiologic and Atmospheric Oxygen Concentrations

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



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

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.