Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development

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The oxygen concentration used during embryo culture can influence embryo development and quality. Reducing the oxygen concentration in the atmosphere to 2% during post-compaction culture of mouse embryos perturbs embryonic gene expression. This study examined the effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. Embryos were cultured from the zygote to morula stage under 7% oxygen, followed by 20, 7 or 2% oxygen to the blastocyst stage. Cultured and *in vivo* developed blastocysts were transferred into pseudopregnant recipients. Fetal and placental outcomes were analysed at day 18 of pregnancy. Implantation rate was not influenced by embryo culture conditions, but resorption rates were increased in embryos cultured under 2% oxygen, compared with 7% oxygen. Day 18 fetal weights were reduced following culture under 2%, compared with 7 or 20% oxygen, or *in vivo* development. Placental weight was not influenced by culture conditions. No differences in the proportion of junctional or labyrinthine exchange regions within the placenta or the morphometry of the labyrinthine region were detected. Surface density (surface area/gram labyrinth) of trophoblast available for exchange was reduced in placentas developed from embryos cultured under 2% oxygen, compared with 7% oxygen. Placental gene expression of *Slc2a1*, *Slc2a3*, *Igf2*, *Igf2r* and *H19* was not influenced by oxygen conditions during embryo culture. Thus, exposure to 2% oxygen during post-compaction pre-implantation embryo development has adverse consequences for fetal development in the mouse. Oxygen is a significant component of the embryonic environment and reductions in oxygen availability can influence both embryonic gene expression and subsequent fetal development.

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Oxygen is an important component of oviductal and uterine environments and may have important roles in regulating embryonic development. Oviductal oxygen levels range from 35 to 60 mmHg (5–8%) in the rabbit, rodent and rhesus monkey, while the uterine environment has a lower oxygen tension of 11–14 mmHg (1.5–2%) in the rhesus monkey (Fischer & Bavister, 1993). Oxygen levels in the uterus are cycle dependent and decrease at the time of implantation, when in rabbits and hamsters a decrease from 5.3% to 3.5% oxygen is observed (Fischer & Bavister, 1993). Lowering the oxygen concentration in the gaseous phase during embryo culture, from atmospheric levels to more physiological levels, has been associated with improved embryo development, in terms of blastocyst development rate and embryo cell number, in a number of species, supporting the suggestion that reduced oxygen atmospheres are beneficial for embryo development (Quinn & Harlow, 1978; Pabon *et al.* 1989; Umaoka *et al.* 1992; Li & Foote, 1993; Gardner & Lane, 1996; Dumoulin *et al.* 1999; Thompson *et al.* 1990, 2000; Hooper *et al.* 2001; Orsi & Leese, 2001). In the mouse, increased blastocyst development rates and/or cell numbers have been reported following culture under 7% (Gardner & Lane, 1996) or 5% oxygen (Quinn & Harlow, 1978; Pabon *et al.* 1989; Umaoka *et al.* 1992; Hooper *et al.* 2001; Orsi & Leese, 2001), when compared with 20% oxygen, in both F1 hybrid and outbred strains.

In addition, embryos appear to encounter a decreasing oxygen gradient as they travel from the oviduct to the uterus (Fischer & Bavister, 1993), with post-compaction

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blastocyst development occurring in the lower oxygen environment of the uterus. Exposure to a lower oxygen environment *in vitro* may be most beneficial during the post-compaction period. Decreasing the oxygen concentration, from 7 to 2%, during the compaction to blastulation stages of bovine embryo *in vitro* production, increases the development rate of grade 1 and 2 blastocysts (Thompson *et al.* 2000). Furthermore, this suggests that, in the cow, a 2% oxygen environment during the post-compaction period is beneficial for *in vitro* development. However, our studies suggest that the response of the embryo to oxygen environments less than 5% varies between species (Harvey *et al.* 2004; Kind *et al.* 2005). Culture of mouse embryos under 2% oxygen, during the post-compaction period, does not alter blastocyst development rates, when compared with 7 or 20% oxygen (Kind *et al.* 2005). However, mouse embryos cultured post-compaction under 2% oxygen have 3- to 4-fold higher levels of expression of the oxygen-regulated genes *Slc2a1* (formerly known as *Glut-1*), *Slc2a3* (formerly known as *Glut-3*) and *Vegf*, when compared with embryos cultured under 7 or 20% oxygen, or embryos developed *in vivo* (Kind *et al.* 2005). This contrasts with the bovine embryo, where *Slc2a1* expression is increased by 30–50% in bovine embryos cultured at 2% oxygen post-compaction, when compared with embryos cultured under 7 or 20% oxygen, but is not different from *in vivo* developed embryos (Harvey *et al.* 2004). Thus, 2% oxygen represents a hypoxic environment for the mouse blastocyst, and is associated with perturbations in gene expression.

Perturbed gene expression in the mouse embryo in response to a low oxygen environment raises the question of whether this may be associated with longer-term consequences for subsequent development. Altered embryonic environments, including suboptimal culture conditions, have been implicated in adverse fetal and placental developmental outcomes (Fleming *et al.* 2004; Sjöblom *et al.* 2005). Therefore, the aim of this study was to determine the effects of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. In addition, placental morphometry and gene expression were examined to further investigate placental development.

Methods

Culture media

For embryo culture, potassium simplex optimised media (KSOM) (Erbach *et al.* 1994) containing 0.2 mM glucose and modified to contain 0.05 mM phosphate (Biggers & McGinnis, 2001) and 100 μM EDTA was used (Kind *et al.* 2005). Fatty acid-free BSA was purchased from ICPbio, Auckland, New Zealand. L-Alanyl-L-glutamine (1.0 mM) (GlutaMax, Invitrogen, Carlsbad, CA, USA) was substituted for glutamine. All other reagents were from Sigma Chemical Company.

Embryo collection and culture

Female 3- to 4-week-old CBAB6F1 mice were injected subcutaneously at 16.00 h with 5 IU of equine chorionic gonadotropin (Folligon, Intervet, Boxmeer, Holland), followed 48 h later by 5 IU of human chorionic gonadotropin (hCG) (Pregnyl, Organon, Sydney, Australia). Following hCG injection, females were caged individually, overnight with a male of the same strain. Twenty-two hours post hCG females were humanely killed by cervical dislocation, and 1-cell embryos were collected by flushing the oviducts into Hepes-buffered handling media, containing 50 U ml^{-1} hyaluronidase. Cumulus cells were removed and embryos were washed twice in handling media and once in KSOM media. All procedures were approved by the Animal Ethics Committee, University of Adelaide.

Embryos were cultured in Petri dishes in 20 μl drops of modified KSOM medium under mineral oil in 37°C humidified atmospheres at 7% O_2, 6% CO_2, 87% N_2 in modular incubator chambers (Billups-Rothenburg, Del Mar, CA, USA). Fifteen embryos were included per drop and embryos were randomly assigned to one of three separate Petri dishes in each experiment. After 48 h culture (~72 h post hCG), when embryos had reached the morula stage, dishes were transferred to 2, 20 or 7% O_2, containing 6% CO_2 and the balance N_2, in modular incubators. Embryos were cultured for a further 40 h (~112 h post hCG), to the blastocyst stage. *In vivo* developed blastocysts were collected from superovulated mice at 88 h post hCG injection.

Embryo transfers

Naturally ovulating female Swiss Albino mice (10–12 weeks of age), mated with vasectomised CBAB6F1 males, were used as recipients for embryo transfers. Mating was confirmed by the presence of a vaginal plug the following morning (day 1 of pseudopregnancy). Blastocyst transfers were performed on day 4 of pseudopregnancy. Recipients were anaesthetized by intraperitoneal injection of 2% Avertin (2,2,2-Tribromoethanol in 2-methyl-2-butanol, diluted to 2% solution in H_2O; 0.015 ml (g body weight)$^{-1}$, 0.3 mg (g body weight)$^{-1}$), and level of anaesthesia was assessed by suppression of the withdrawal reflex and monitoring respiration rate. Blastocysts from one of the three oxygen treatment groups, or *in vivo* derived blastocysts, were randomly allocated to individual uterine horns of the female recipient, with six blastocysts transferred to each horn.

Fetal and placental analysis at day 18 of pregnancy

Embryo transfer recipients were humanely killed on day 18 of pregnancy by cervical dislocation. Number of viable or resorbing implantation sites was recorded. Fetuses and placentas were weighed and fetal crown–rump length was measured. Placentas were either immediately snap-frozen for later gene expression analysis, or fixed in 4% paraformaldehyde–2.5% polyvinyl pyrrolidone in 70 mM sodium phosphate buffer (pH 7.4) overnight at 4°C. The following day placentas were bisected sagittally, washed in four changes of PBS over 2 days, and processed and embedded in paraffin wax with cut surfaces down.

Thirty serial mid-sagittal 7 μm sections were cut and placed on 10 3-aminopropyltriethoxysilane-coated slides and the first slide with a full placental face was stained with Masson's trichrome, as previously described (Roberts *et al.* 2001, 2003; Sjoblom *et al.* 2005). Sections were examined on an Olympus BH2 microscope equipped with a Video Image Analysis system using Video Pro software (Leading Edge, Adelaide, Australia). The total cross-sectional areas of junctional and labyrinthine zones were measured, and expressed as a percentage of the total midsaggital area. Placentas from *in vivo* ($n = 9$), 20% ($n = 11$), 7% ($n = 13$) and 2% ($n = 13$) groups from 5 to 9 dams were analysed.

Placental labyrinthine morphometry

Placental sections were double immunolabelled, as previously described (Roberts *et al.* 2001, 2003; Sjoblom *et al.* 2005), with rat anti-mouse monoclonal antibodies MTS12 (kind gift of Professor Richard Boyd, Department of Pathology and Immunology, Monash University, Melbourne, Australia), to label murine fetal endothelial cells, and rabbit anti-human pan-cytokeratin (Zymed, San Francisco, CA, USA) to label trophoblast. A triple layer technique was used as previously described (Roberts *et al.* 2003; Sjoblom *et al.* 2005). Sections were deparaffinized and brought to water. Sections were incubated at 37°C for 15 min in 0.03% protease (Sigma) for antigen retrieval. Endogenous peroxidase activity was quenched by incubating with 6% hydrogen peroxide for 40 min, then sections were washed in three changes of PBS for 5 min each. Non-specific binding was blocked by incubation with phosphate-buffered saline containing 10% swine serum and 1% BSA (Sigma) for 10 min without washing.

Sections were then incubated overnight in a humidified chamber at room temperature with MTS-12 supernatant with 10% swine serum and 1% BSA. Sections were washed as before and incubated for 60 min in biotinylated rabbit anti-rat secondary antibody (DakoCytomation, Denmark; 1 : 200 in PBS containing 10% swine serum and 1% BSA). Sections were washed again and streptavidin–horseradish peroxidase (Zymed) was added for 60 min. Following washing, sections were incubated with diaminobenzidine containing 2% ammonium nickel (II) sulphate (Sigma), to form a black precipitate.

Sections were washed as before and then incubated overnight as above with rabbit anti-human pan-cytokeratin diluted 1 : 100 in PBS containing 10% swine serum and 1% BSA. The entire process was repeated as before, except that a biotinylated goat anti-rabbit antibody (1 : 200, in PBS containing 10% swine serum and 1% BSA) was used, and nickel was omitted from the chromogen, leaving a brown precipitate. Negative controls, omitting the primary antibody, were included in all analyses.

The sections were examined on an Olympus Vanox AHBT3 microscope with a 2.5 × ocular lens and 20 × objective lens, equipped with a Video Image Analysis System with IP Laboratory Image analysis program. Volume densities of trophoblast, fetal capillaries and maternal blood space in the labyrinth were quantified by point counting with an isotropic L-36 Merz transparent grid placed on the monitor screen (Weibel, 1979). Ten fields (360 points) were counted in randomly selected sections for each placenta. The first field was chosen randomly and adjacent sections were systematically selected 0.5 mm apart using a stage micrometer. The volume density of each labyrinthine component was calculated as volume density, $V_d = P_a/P_T$, where P_a is the total number of points falling on that component and P_T is the total number of points counted (Weibel, 1979; Roberts *et al.* 2001, 2003). The surface density (surface area per gram of placenta) of trophoblast was measured by line intercept counting on the same grid and the same fields, and calculated taking into account the total magnification on the monitor using the formula: surface density, $S_v = 2 \times I_a/L_T$, where I_a is the number of intercepts with the line, and L_T is the total length of the lines applied (Weibel, 1979). The arithmetic mean barrier to diffusion was calculated as B_T (barrier thickness) $= V_d/S_V$, where V_d is the volume density of trophoblast, and S_v is the surface density of trophoblast. Mass of labyrinthine tissue (M_L) was calculated as percentage labyrinth × placental weight (g). Surface area of trophoblast was then calculated according to the formula $SA = S_V \times M_L$ cm^2. Placentas from *in vivo* ($n = 9$), 20% ($n = 10$), 7% ($n = 13$) and 2% ($n = 11$) from 5 to 9 dams per group were analysed.

Placental gene expression

Frozen placentas were bisected and total RNA was extracted from half of each placenta using TriReagent (Sigma), according to the manufacturer's instructions. Final RNA pellets were redissolved in 50 μl sterile water, and samples were DNAse treated (DNA-free, Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA concentration was calculated from the absorbance at A_{260}.

Table 1. Primer sequences used for real time RT-PCR

Gene	GenBank accession no.	Primer sequence	Amplicon size
Igf2	U71085	Forward – AAGAGTTCAGAGAGGCCAAACG Reverse – CACTGATGGTTGCTGGACATCT	103 bp
Igf2r	NM_010515	Forward – TTTTGGGCGCCTTGCAT Reverse – AGGGCAAGGATCACCATTCAC	88 bp
Slc2a1	M23384	Forward – CCAGCTGGGAATCGTCGTT Reverse – CAAGTCTGCATTGCCCATGAT	76 bp
Slc2a3	X61093	Forward – CTCTTCAGGTCACCCAACTACGT Reverse – CCGCGTCCTTGAAGATTCC	121 bp
Rn18s	AF176811	Forward – AGAAACGGCTACCACATCCAA Reverse – CCTGTATTGTTATTTTTCGTCACTACCT	91 bp

Total RNA (2 μg) from each placenta was reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen). RNA was combined with 250 ng random primers and 500 μM of each dNTP (Invitrogen) and incubated at 65°C for 5 min, followed by incubation on ice for 1 min. Reverse transcription was then performed in a final volume of 20 μl, containing First-Strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT, and 200 U Superscript III. Reactions were incubated at 25°C for 10 min, 50°C for 60 min and 70°C for 15 min. cDNAs were stored at −20°C until use. RNA from an *in vivo* developed day 18 placenta was used to generate a pooled standard cDNA. Ten 2 μg RNA aliquots were reverse transcribed, pooled, then diluted to the equivalent of cDNA generated from 25 ng starting RNA per microlitre and stored as aliquots at −20°C.

Real-time PCR was performed in an Applied Biosystems 5700 GeneAmp Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were designed using Primer Express (Applied Biosystems) and were synthesized by GeneWorks, Adelaide, Australia. Primer sequence details are described in Table 1. PCR was performed in 20 μl volumes containing 1 × SYBR Green Master Mix (Applied Biosystems) and 500 nM forward and reverse primers. cDNAs were diluted in water to a concentration per microlitre equivalent to cDNA generated from 0.5 ng starting RNA. For *Igf2*, 2 μl diluted cDNA was analysed (1 ng equivalent); for *Igf2r*, *H19*, *Slc2a1* and *Slc2a3*, 4 μl diluted cDNA was analysed (2 ng equivalent) and for *Rn18S*, 1 μl diluted cDNA was analysed (0.5 ng). The thermal cycling program consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A cycle threshold (C_t) was calculated for each sample using the GeneAmp 5700 software. Dissociation curves, to detect non-specific amplification, were generated for all reactions, and the presence of a single amplified product was also confirmed by gel electrophoresis. No template samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification products. Placentas from *in vivo*, 2% and 20% (all $n = 10$) and 7% ($n = 11$) groups from 6 to 8 mothers per group were analysed.

Statistics

All data are presented as mean ± S.E.M. Implantation and fetal development data were analysed by GLIM statistical package (version 4.0, National Algorithm Group, Oxford, UK) by general linear modelling using the log likelihood statistic. All other statistical analyses were carried out using SPSS for Windows (SPSS, Chicago). All comparisons between treatment groups were performed using one-way ANOVA. Litter size was included as a covariate in analysis of fetal and placental outcome measures as indicated in the text. Bonferroni *post hoc* tests were used for all comparisons between groups. Significance was accepted at $P < 0.05$.

Results

Embryo development rates

Embryo development rates were assessed following culture for 48 h (development to morula) and 88 h (development to blastocyst) (Table 2). There were no differences between groups in overall development to the morula or blastocyst stages. Exposure to differing oxygen concentration during the post-compaction period did not alter development rates from morula to blastocyst (Table 2).

Effect of oxygen on pregnancy outcome

Embryos were transferred to a total of 39 recipients. Twenty-seven of the recipients were pregnant in at least one horn at day 18 of pregnancy. Non-pregnant animals contained embryos from all treatment groups (2%: $n = 7$ horns; 7%: $n = 7$ horns; 20%: $n = 8$ horns; *in vivo*: $n = 2$ horns). These animals were not included in further analysis. In addition, two uterine horns that exhibited uterine adhesions were excluded from analysis. Therefore, a total of 52 uterine horns were studied (2%: $n = 15$;

Table 2. Effect of oxygen concentration used during the postcompaction period of embryo culture on embryo development rates

		% Development		
Oxygen concentration	Number of 1-cell embryos	to morula	to blastocyst	morula to blastocyst
20%	477	94 ± 2	91 ± 2	97 ± 1
7%	431	94 ± 1	90 ± 2	96 ± 2
2%	500	91 ± 3	84 ± 4	92 ± 2

All embryos were cultured under 7% oxygen to the morula stage. Data expressed as mean ± S.E.M.

Table 3. The effect of post-compaction oxygen concentration on implantation rates and subsequent fetal and placental development

	2% O_2	7% O_2	20% O_2	*In vivo*
Implantation rate[A] (%)	65.5 ± 6.6	80.3 ± 7.0	73.8 ± 8.5	69.4 ± 10.9
Viable fetuses/blastocyst implanted[B] (%)	55.6 ± 10.5[a]	79.2 ± 9.2[b]	66.4 ± 9.5[a,b]	88.9 ± 7.0[a,b]
Fetal weight (mg)	857 ± 22[a] (30)	944 ± 14[b] (40)	956 ± 19[b] (38)	981 ± 27[b] (20)
Fetal crown–rump length (mm)	18.2 ± 0.3[a] (30)	19.1 ± 0.2[a,b] (40)	19.3 ± 0.2[b] (38)	19.9 ± 0.3[b] (20)
Placental weight (mg)	91.2 ± 3.5 (25)	85.9 ± 3.9 (35)	89.1 ± 2.9 (32)	95.3 ± 3.8 (19)
Fetal : placental weight ratio	9.7 ± 0.4[a]	11.7 ± 0.6[b]	11.3 ± 0.4[b]	10.9 ± 0.3[ab]

Data expressed as mean ± S.E.M.; values in parentheses are number of fetuses or placentas. [A]Implantation rate: number of fetal/placental units or resorptions present as a percentage of the number of embryos transferred. [B]Viable fetuses/blastocyst implanted: percentage of implanted embryos that developed into a viable fetus. Number of uterine horns per treatment group, 6 embryos transferred per horn, 2%: $n = 14$; 7%: $n = 11$; 20%: $n = 14$; *in vivo*: $n = 6$. Different superscripts within a row represent statistically significantly differences, $P < 0.05$.

7%: $n = 14$; 20%: $n = 15$; *in vivo*: $n = 8$). No implantation sites were detected in seven horns (2%: $n = 1$; 7%: $n = 3$; 20%: $n = 1$; *in vivo*: $n = 2$). Implantation rates were not different between culture groups or when compared with *in vivo* derived blastocysts (Table 3). However, development of a viable fetus following implantation (viable fetuses/blastocyst implanted) was reduced when embryos were cultured under 2% oxygen, compared with 7% oxygen (Table 3).

Viable conceptuses present within a uterine horn at day 18 of pregnancy ranged from 1 to 6 (mean, 3.4 ± 0.2). Total litter size carried by the recipient mothers ranged from 1 to 10 (mean, 4.9 ± 0.4). The number of viable fetuses at day 18 of pregnancy within a horn did not vary with treatment (2%, 3.2 ± 0.4 ($n = 10$ horns); 7%, 4.0 ± 0.4 ($n = 10$); 20%, 2.9 ± 0.5 ($n = 13$); *in vivo*, 3.7 ± 0.6 ($n = 6$)). Total litter size carried by the mother was also calculated to provide an indication of total maternal load (mothers carrying a horn containing 2%: litter size, 5.4 ± 0.6, 7%: 6.5 ± 0.7; 20%: 4.9 ± 0.8; *in vivo*: 4.0 ± 0.6).

Effect of oxygen concentration on fetal and placental weights

Fetal weight was reduced following embryo culture at 2% oxygen post-compaction, compared with culture at 7 or 20% oxygen (Table 3). Culture under 2% oxygen also reduced fetal weight when compared with embryos developed *in vivo* (Table 3). Culture under 7 or 20% oxygen did not alter fetal weight when compared with *in vivo* development (Table 3). Fetal crown–rump length was also reduced following embryo culture under 2% oxygen, when compared with 20% oxygen, or *in vivo* development (Table 3). Differences in fetal size remained significant when corrected for number of fetuses within the treatment horn, or total litter size for the mother (combined number of fetuses for both horns).

There was no difference in placental weights across all groups (Table 3). Fetal : placental weight ratios in the 2% oxygen treatment group were reduced when compared with 7 or 20% cultured embryos, but were not different from *in vivo* developed embryos (Table 3).

Effect of oxygen on placental structure

Oxygen concentration used during embryo culture did not significantly alter the mid-sagittal cross sectional total area or the proportion of labyrinthine or junctional zone of the placenta (Table 4).

The volume density (proportion) of trophoblast cells, fetal capillaries or maternal blood space within the labyrinthine region was not influenced by embryo culture,

Table 4. The effect of post-compaction oxygen concentration on placental structure at day 18 of pregnancy

	2% O_2	7% O_2	20% O_2	*In vivo*
Total area (mm^2)	10.3 ± 0.4	9.4 ± 0.4	10.1 ± 0.3	9.2 ± 0.7
Proportion junctional zone (%)	45.0 ± 1.8	44.5 ± 1.4	47.3 ± 2.1	43.4 ± 1.6
Proportion labyrinth (%)	55.0 ± 1.8	55.5 ± 1.4	52.7 ± 2.1	56.6 ± 1.6
Labyrinth to junctional zone ratio	1.26 ± 0.09	1.28 ± 0.08	1.16 ± 0.11	1.33 ± 0.09

2% ($n = 13$), 7% ($n = 13$), 20% ($n = 11$), *in vivo* ($n = 9$) placentas from 5 to 9 mothers per treatment. Data expressed as mean ± S.E.M.

Table 5. The effect of post-compaction oxygen concentration on placental labyrinth morphometry at day 18 pregnancy

	2% O_2	7% O_2	20% O_2	*In vivo*
Volume density trophoblast (%)	$64.5 \pm 1.2\%$	$64.4 \pm 1.0\%$	$62.4 \pm 1.4\%$	$63.4 \pm 1.4\%$
Volume density maternal blood space (%)	$16.3 \pm 0.9\%$	$17.2 \pm 0.9\%$	$17.8 \pm 0.8\%$	$16.6 \pm 1.3\%$
Volume density fetal capillaries (%)	$19.2 \pm 1.3\%$	$18.4 \pm 0.8\%$	$19.8 \pm 1.5\%$	$20.0 \pm 1.5\%$
Surface density of trophoblast (surface area cm^2 (g labyrinth)$^{-1}$)	766.2 ± 38.5^a	899.5 ± 35.4^b	$851.0 \pm 22.9^{a,b}$	$846.3 \pm 34.4^{a,b}$
Trophoblast barrier thickness (μm)	8.70 ± 0.57	7.30 ± 0.34	7.37 ± 0.22	7.60 ± 0.35
Total surface area of trophoblast for exchange (cm^2)	39.2 ± 2.8	44.0 ± 3.0	40.0 ± 1.8	44.6 ± 3.5

2% ($n = 11$), 7% ($n = 13$), 20% ($n = 10$), *in vivo* ($n = 9$) placentas from 5 to 9 mothers per treatment. Data expressed as mean ± S.E.M. Volume densities are proportions of each component within the placental labyrinth. Different superscripts within a row represent statistically significantly differences, $P < 0.05$.

or the oxygen concentration used during embryo culture (Table 5). Surface density (surface area per gram labyrinth) of trophoblast apical membrane available for exchange in the placental labyrinth, was reduced in placentas developed from embryos cultured at 2% oxygen, when compared with culture at 7% oxygen, with or without correction for litter size (Table 5). However, surface density of trophoblast in placentas from embryos cultured at 2% oxygen was not different when compared with those from embryos cultured at 20% oxygen or developed *in vivo* (Table 5). Trophoblast barrier thickness tended to be increased in placentas from embryos cultured under 2% oxygen ($P = 0.06$ overall ANOVA, Table 5). Total surface area of trophoblast for exchange was not altered by embryo culture conditions (Table 5).

Effect of oxygen on placental gene expression

No differences were observed in expression of *Slc2a1, Slc2a3, Igf2, Igf2r* or *H19*, in day 18 placentas developed from embryos cultured under 2, 7 or 20% oxygen, or developed *in vivo* and transferred into pseudopregnant recipients (Fig. 1).

Discussion

This study demonstrates that *in vitro* exposure of mouse embryos to 2% oxygen, throughout the post-compaction period, has adverse consequences for subsequent fetal growth. We have previously demonstrated that 2% oxygen alters the expression of key embryonic genes in the mouse pre-implantation embryo (Kind *et al.* 2005). Oxygen-regulated gene expression in the embryo may be mediated via the transcription factors, the hypoxia-inducible factors (Harvey *et al.* 2004). Thus, under low oxygen conditions the embryo responds to its environment with a transcriptionally mediated alteration in gene expression. This study suggests that these perturbations in gene expression, and potentially associated changes in embryonic metabolism, may contribute to subsequent perturbations in fetal development.

Post-implantation mortality in embryos cultured at 2% oxygen post-compaction was increased and the weight of viable fetuses was reduced following embryo culture at 2% oxygen. Thus, although similar numbers of embryos cultured under different oxygen conditions developed to blastocyst, perturbations associated with exposure to low oxygen impact on post-implantation viability. Reduced fetal weights have been observed previously in the mouse following embryo culture (Harlow & Quinn, 1979; Khosla *et al.* 2001*a*; Sjoblom *et al.* 2005). However, fetal weight was not reduced in embryos cultured at 7 or 20% oxygen in the current study, when compared with *in vivo* developed embryos. Others have reported a significant decrease in fetal development per blastocyst transferred and fetal development per implantation site when embryos were cultured at 20%, compared with 5% oxygen (Karagenc *et al.* 2004). However, unlike the current study, the exposure to different oxygen conditions commenced at

the 1-cell stage in that study (Karagenc *et al.* 2004). Previous studies have demonstrated reduced blastocyst development rates when embryos are cultured under 20%, compared with 5 or 7% oxygen from the zygote stage (Pabon *et al.* 1989; Gardner & Lane, 1996; Orsi & Leese, 2001). Similarly, culture under 20% oxygen reduces blastocyst total cell number when commenced at the zygote or 2-cell stage, but not the 8-cell stage (Karagenc *et al.* 2004). Inner cell mass number was reduced by culture at 20%, compared with 5% oxygen, regardless of when the culture commenced, although these effects were most significant with treatment from the 1-cell stage (Karagenc *et al.* 2004). Nevertheless, no difference in fetal weights at day 15 gestation were observed following embryo culture for 95 h under 20%, compared with 5% oxygen (Karagenc *et al.* 2004). Similarly, culture of embryos from the 2-cell stage to blastocyst under 20% or 5% oxygen was not associated with differences in fetal or placental weight at day 18 of pregnancy (Harlow & Quinn, 1979).

Successful implantation and development of mouse embryos following exposure to oxygen concentrations ranging from 2 to 20% *in vitro* demonstrates that the pre-implantation embryo is able to adapt to significant variation in environmental conditions. Indeed, no difference in fetal weight was observed when embryos were cultured at 7 or 20% oxygen, or developed *in vivo* and then transferred. Variations in oxygen concentration were imposed during the post-compaction period in the current study, and the ability of the embryo to adapt to environmental perturbations, including oxygen, may be greater in the post- compared with precompaction embryo (Karagenc *et al.* 2004; Lane & Gardner, 2005; Zander *et al.* 2006). Increased expression of key genes in the blastocyst under low oxygen (Kind *et al.* 2005) demonstrates that the embryo can detect and respond to environmental change, and may be one mechanism through which the embryo acts to maintain metabolic and cellular homeostasis when exposed to perturbed environments (Lane & Gardner, 2005). Nevertheless, post-implantation viability was decreased by 2% oxygen culture, and while remaining embryos implanted and successfully underwent fetal and placental development, fetal, although not placental, weight was decreased. Whether these perturbations of fetal growth and development are associated with longer-term consequences for adult health (Barker, 1998; Gluckman & Hanson, 2004; McMillen & Robinson, 2005) remains to be determined.

Perturbations in embryonic metabolism may be one mechanism through which low oxygen influences embryonic viability and subsequent development. Alterations in gene expression in blastocysts cultured under 2% oxygen include a 3- to 4-fold increase in the expression of *Slc2a1* (*Glut-1*) and *Slc2a3* (*Glut-3*)

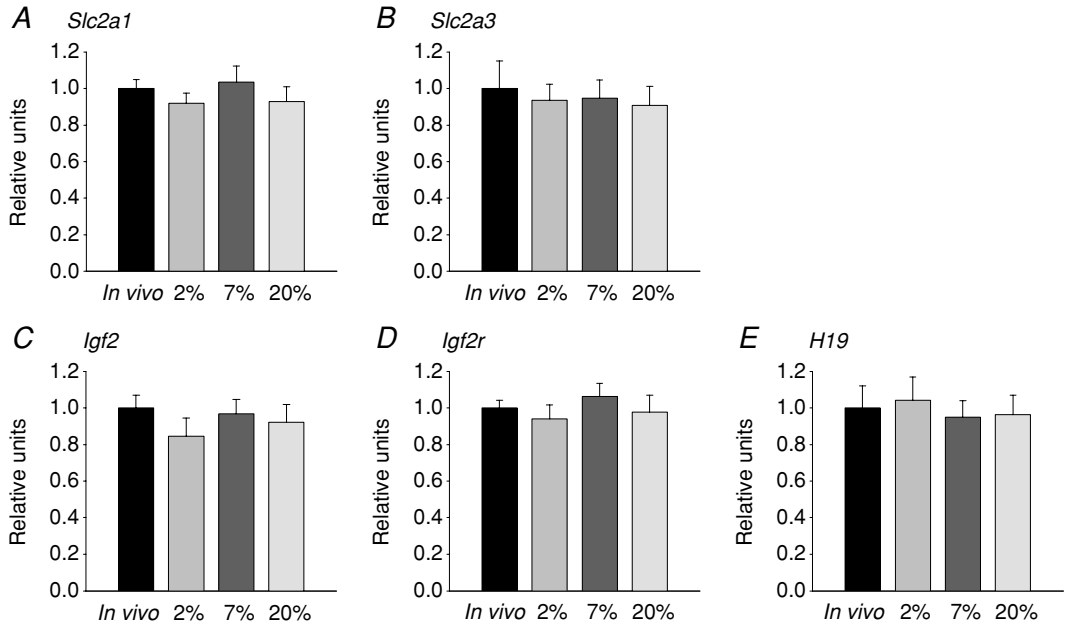

Figure 1. Gene expression
Expression of, *Slc2a1* (*A*), *Slc2a3* (*B*), *Igf2* (*C*), *Igf2r* (*D*) and *H19* (*E*) in day 18 mouse placenta developed from embryos exposed to 7% oxygen from 1-cell to morula, followed by 20, 7 or 2% oxygen from morula to blastocyst, or developed *in vivo* to the blastocyst stage, and then transferred. All results are expressed as a fold change from the *in vivo* developed group and have been normalized to *Rn18s* expression. Results are mean ± S.E.M. from 10 placentas (*in vivo*, 2%, 20%) and 11 placentas (7%) from 6 to 8 litters per treatment.

(Kind *et al.* 2005) and glucose uptake is increased in embryos cultured under 2% oxygen, when compared with culture at 7% oxygen (Kind *et al.* 2005). Low oxygen also stimulates the expression of glycolytic enzymes (Semenza, 1999), although this has not been examined in the blastocyst. It has been suggested that up-regulation of embryonic metabolism is associated with a reduction in viability of the embryo (Leese, 2002). Mouse blastocysts with elevated glycolytic activity have a reduced ability to develop into a viable fetus (Lane & Gardner, 1996). However, higher rates of glucose uptake by the blastocyst are also associated with an increase in successful fetal development (Lane & Gardner, 1996; Gardner *et al.* 2001). Metabolic changes associated with culture under low oxygen, in the current study, may influence subsequent development; however, more complete analysis of metabolism in embryos cultured under 2% oxygen is required to assess this further. Blastocyst cell number is also associated with subsequent fetal viability (Lane & Gardner, 1997). Embryos cultured under 2% oxygen post-compaction have a reduction in total cell number in the blastocyst (Kind *et al.* 2005), which may also contribute to deficits in fetal development.

No significant difference in placental weights was observed after culture at different oxygen concentrations. However, fetal : placental weight ratio was reduced by post-compaction embryo culture at low oxygen. Previous studies have reported a decrease in the relative proportion of the placental labyrinth, the region responsible for exchange of substrates in the mouse placenta, and an increase in the relative proportion of junctional zones in placentas developed from cultured embryos (Sjoblom *et al.* 2005). No difference in the proportion of labyrinthine and junctional zones in the placenta following embryo culture was observed in the current study. Surface density of the trophoblast apical membrane available for exchange between maternal and fetal blood in the labyrinth was lowest in placentas from 2% cultured embryos, although this was only significantly different from placentas developed following embryo culture under 7% oxygen, and not when compared with *in vivo* developed embryos. Similarly, arithmetic mean barrier thickness of the trophoblast tended to be highest in placentas from 2% oxygen-cultured embryos but this difference was not significant. In contrast, others have reported reduced thickness and increased surface density of the trophoblast layer following *in vitro* embryo culture, when compared with *in vivo* developed embryos (Sjoblom *et al.* 2005). However, these results were obtained in a culture system utilizing 20% oxygen (Sjoblom *et al.* 2005). No differences were observed in placentas from embryos cultured at 20% oxygen, when compared with those developed *in vivo*, in the current study; however, other components of the embryo culture system may also influence placental development (Sjoblom *et al.* 2005). Thus, the current study suggests that alterations in placental structure may be induced by culture under different oxygen conditions; however, further analysis is required to assess this. In addition, the contribution of these changes to the decrease in fetal weight, observed following culture under low oxygen conditions, requires further investigation.

Expression of a selection of key placental genes was also examined in the current study. Placental expression levels of *Igf2, Igf2r, H19* and the glucose transporter genes, *Slc2a1* and *Slc2a3* were not altered at day 18 following embryo culture under different oxygen conditions. A role for SLC2A1 (GLUT-1) in taking up glucose for use as a placental fuel, and for SLC2A3 (GLUT-3) in glucose transfer to the fetus has been suggested in the rodent placenta (Zhou & Bondy, 1993). IGF2 has been identified as an important regulator of placental growth (DeChiara *et al.* 1990; Baker *et al.* 1993; Constancia *et al.* 2002). Deletion of the *Igf2* gene (De Chiara *et al.* 1990; Baker *et al.* 1993), or a placenta-specific transcript of *Igf2*, expressed predominantly in labryrinthine trophoblast (Constancia *et al.* 2002), is associated with retarded placental growth, and altered placental transfer function in mice (Sibley *et al.* 2004). In the current study, total *Igf2* expression was measured, and analysis of expression from different promoters may be of further interest. In addition, *Igf2, Igf2r* and *H19* are imprinted genes. Disruption of *H19* imprinting has been reported in mouse blastocysts (Doherty *et al.* 2000; Mann *et al.* 2004), fetuses (Khosla *et al.* 2001*a*) and in the placenta (Mann *et al.* 2004) following culture of embryos in Whitten's medium (Doherty *et al.* 2000; Mann *et al.* 2004) or in the presence of serum (Khosla *et al.* 2001*a*). Similarly, disruption of imprinting of the *Igf2, Igf2r* and *H19* gene, and altered expression of the P0 specific *Igf2* transcript have been reported in association with placental overgrowth in placentae from cloned mice (Ogawa *et al.* 2003). In the current study no differences in expression levels of these genes were observed. However, specific methylation analysis is required to confirm the absence of imprinting defects. Nevertheless, it is possible that insults imposed on the embryo during the post-compaction period only, such as the low oxygen exposure in the current study, exert their effects on subsequent development through mechanisms such as altered gene expression, rather than perturbations in imprinting or epigenetic mechanisms.

Therefore, the current study has demonstrated that reducing the oxygen concentration used during the post-compaction period of embryo culture, to a level that represents a hypoxic stimulus for the mouse embryo, is associated with increased post-implantation mortality and reduced fetal weight. Although the mouse embryo was able to adapt to significant variation in oxygen concentration with no effect on placental weight or gene expression, fetal weight was decreased and some measures of placental structure were altered following embryo culture under

2% oxygen. Altered fetal development has been associated with adverse adult health outcomes, including increased risk of cardiovascular and metabolic diseases (Barker, 1998; Gluckman & Hanson, 2004; McMillen & Robinson, 2005). This study further supports the suggestion that perturbation of the embryonic environment can influence subsequent fetal, and possibly placental development, and thus may also have longer-term consequences for adult health (Khosla *et al.* 2001*b*; Ecker *et al.* 2004; Fleming *et al.* 2004; Sjoblom *et al.* 2005). The current results indicate that oxygen is an important component of the embryonic environment, and demonstrates that reductions in the oxygen level experienced by the embryo during the post-compaction period can alter both embryonic gene expression (Kind *et al.* 2005) and subsequent fetal development.

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