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Maternal chronic hypoxia increases expression of genes regulating lung liquid movement and surfactant maturation in male fetuses in late gestation

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Key points

- Chronic fetal hypoxaemia is a common pregnancy complication associated with intrauterine growth restriction that may influence respiratory outcome at birth.
- We investigated the effect of maternal chronic hypoxia for a month in late gestation on signalling pathways regulating fetal lung maturation and the transition to air-breathing at birth using isobaric hypoxic chambers without alterations to maternal food intake.
- Maternal chronic hypoxia in late gestation increases fetal lung expression of genes regulating hypoxia signalling, lung liquid reabsorption and surfactant maturation, which may be an adaptive response in preparation for the successful transition to air-breathing at birth.
- In contrast to other models of chronic fetal hypoxaemia, late gestation onset fetal hypoxaemia promotes molecular regulation of fetal lung maturation. This suggests a differential effect of timing and duration of fetal chronic hypoxaemia on fetal lung maturation, which supports the heterogeneity observed in respiratory outcomes in newborns following exposure to chronic hypoxaemia *in utero*.

Abstract Chronic fetal hypoxaemia is a common pregnancy complication that may arise from maternal, placental and/or fetal factors. Respiratory outcome of the infant at birth likely depends on the duration, timing and severity of the hypoxaemic insult. We have isolated the effect of maternal chronic hypoxia (MCH) for a month in late gestation on fetal lung development. Pregnant ewes were exposed to normoxia (21% O_2) or hypoxia (10% O_2) from 105 to 138 days of gestation (term ∼145 days). At 138 days, gene expression in fetal lung tissue was determined by quantitative RT-PCR. Cortisol concentrations were determined in fetal plasma and lung tissue. Numerical density of surfactant protein positive cells was determined by immunohistochemistry. MCH reduced maternal P_{aO_2} (106 ± 2.9 *vs.* 47 ± 2.8 mmHg) and fetal body weight (4.0 ± 0.4 *vs.* 3.2 ± 0.9 kg). MCH increased fetal lung expression of the anti-oxidant marker *CAT* and decreased expression of the pro-oxidant marker *NOX-4*. MCH increased expression of genes regulating hypoxia signalling and feedback (*HIF-3α*, *KDM3A*, *SLC2A1*, *EGLN-3*). There was no effect of MCH on fetal plasma/lung tissue cortisol concentrations, nor genes regulating glucocorticoid signalling (*HSD11B-1*, *HSD11B-2*, *NR3C1*, *NR3C2*). MCH increased expression of genes regulating sodium (*SCNN1-B*, *ATP1-A1*, *ATP1-B1*) and water (*AQP-4*) movement in the fetal lung. MCH promoted surfactant maturation (*SFTP-B*, *SFTP-D*, *ABCA3*) at the molecular level, but did not alter the numerical density of surfactant positive cells in lung tissue. MCH in late

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gestation promotes molecular maturation of the fetal lung, which may be an adaptive response in preparation for the successful transition to air-breathing at birth.

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Abbreviations ABCA3, ATP-binding cassette, sub-family A (ABC1), member 3; ACTB, beta-actin; ADM, adrenomedullin; AQP, aquaporin; ATP1, sodium potassium adenosine triphosphatase; CAT, catalase; CLCN2, chloride channel, voltage-sensitive 2; CFTR, cystic fibrosis transmembrane conductance regulator; COL1A1, collagen type 1 alpha 1; EGLN, egl-9 family hypoxia inducible factor; ELN, elastin; GC, glucocorticoid; GPX, glutathione peroxidase; Hb, haemoglobin; HIF, hypoxia inducible factor; HMOX1, heme oxygenase 1; HSD11B, 11β-hydroxysteroid dehydrogenase; IUGR, intrauterine growth restriction; JMJD1A, Jumonji domain containing 1A; KDM3A, lysine (K)-specific demethylase 3A; MCH, maternal chronic hypoxia; NOS, nitric oxide synthase; NOX, nicotinamide adenine dinucleotide phosphate-oxidase; NR3C1, glucocorticoid receptor; NR3C2, mineralocorticoid receptor; PCYT1A, phosphate cytidylyl transferase 1, choline, alpha; PHD, prolyl hydroxylase domain; PPIA, peptidylprolyl isomerase; PR, placental restriction/placentally restricted; qRT-PCR, quantitative real time PCR; RDS, respiratory distress syndrome; SLC2A1, facilitated glucose transporter-1; SCNN1, epithelial sodium channel; SFTP, surfactant protein; SOD, super oxide dismutase; VEGF, vascular endothelial growth factor; YWHAZ, tyrosine 3-monooxygenase.

Introduction

Fetal chronic hypoxaemia is a common pregnancy complication that can predispose to complications at birth and later in life (Hutter & Jaeggi, 2010; Giussani & Davidge, 2013; Giussani, 2016). Exposure to chronic hypoxaemia *in utero* may arise due to a variety of environmental, maternal, placental and/or fetal factors and is commonly associated with intrauterine growth restriction (IUGR) (Morrison, 2008). IUGR is a diverse complication of pregnancy and the developing fetus undergoes a range of neuroendocrine and cardiovascular adaptations as a result of reduced oxygen and nutrient supply that affects normal growth and development (Economides *et al.* 1991; McMillen *et al.* 2001; Dubiel *et al.* 2002). There is heterogeneity in the risk of IUGR newborns developing respiratory complications at birth and this is likely influenced by the specific nature of the altered intrauterine environment encountered during fetal development, in addition to the increased likelihood of being born prematurely. We have identified chronic fetal hypoxaemia as a molecular regulator of lung development which may alter the risk of IUGR newborns developing respiratory complications at birth (McGillick *et al.* 2015, 2016*a*, *b*; Orgeig *et al.* 2015). Many animal models have been used to investigate the effects of IUGR on fetal growth and development, including maternal nutrient restriction, maternal hypoxia and placental insufficiency induced by either uterine artery ligation, uterine carunclectomy, maternal hyperthermia or umbilicoplacental embolisation (McMillen & Robinson, 2005; Morrison, 2008). However, there is limited understanding of the effect of chronic hypoxaemia alone on fetal lung development, as many mammalian models of IUGR associated with chronic hypoxaemia also lead to a reduction in maternal food intake (Robinson *et al.* 1979; Van Geijn *et al.* 1980). Maternal undernutrition including a low protein diet is an effective model to induce IUGR and is itself associated with disturbances of molecular signalling and structural airway and vascular development in the lungs of offspring (Maritz *et al.* 2005; Briana & Malamitsi-Puchner, 2013; Liu *et al.* 2014; Zana-Taieb *et al.* 2015). In this study using sheep, we have isolated the effect of maternal chronic hypoxia (MCH) for a month in late gestation on fetal development using bespoke isobaric hypoxic chambers without alterations to maternal food intake (Allison *et al.* 2016*a*; Brain *et al.* 2015).

Fetal lung development is regulated by a variety of factors that can be influenced by adverse intrauterine conditions. Hypoxia, particularly in prenatal life, is a stimulus for oxidative stress, which occurs due to an imbalance between the generation of reactive oxygen and nitrosative species and the endogenous anti-oxidant system that protects cells from the deleterious effects of free radicals (Giussani *et al.* 2012). Regulation of the pro-oxidant and anti-oxidant balance is therefore essential during pregnancy, as complications associated with oxidative stress have adverse implications for the developing fetus (Suzin *et al.* 2002; Herrera *et al.* 2014). At the cellular level, reduced oxygen tension *in utero* is associated with activation of the hypoxia signalling cascade, regulated by the expression of hypoxia inducible factor (HIF)-α subunits (Benizri *et al.* 2008). Downstream alterations to expression of hypoxia responsive genes have widespread implications on developing organ systems. For instance, in the fetal mouse lung, loss of HIF-α signalling leads to delayed lung maturation and increases the risk of respiratory distress syndrome (RDS) at birth (Compernolle *et al.* 2002). Hypoxia signalling is controlled

by a decrease in HIF-α subunit stability under regulation of the prolyl hydroxylase domain (PHD) enzyme family encoded by the EGLN gene (egl-9 family hypoxia inducible factor), known as *EGLN-1* (PHD-2), *EGLN-2* (PHD-1) and *EGLN-3* (PHD-3) (Bruick & McKnight, 2001). Our studies in a model of early onset chronic hypoxaemia in the placentally restricted (PR) sheep fetus suggest a differential function of the *EGLN*/PHD enzymes during periods of acute *versus* chronic hypoxaemia (Ginouvès *et al.* 2008; Botting *et al.* 2014; Orgeig *et al.* 2015). Hence, alterations to hypoxia signalling *in utero* may underlie altered lung development and downstream risk of IUGR newborns experiencing respiratory complications at birth.

Reabsorption of fetal lung liquid and surfactant maturation are two important processes that enable the lung to function as the primary organ of gas exchange at birth (Avery & Mead, 1959; Hooper *et al.* 2015). Perinatal hypoxaemia may impair the feto-neonatal pulmonary transition leading to neonatal RDS and pulmonary hypertension (Storme *et al.* 2013). However, there is controversy surrounding lung maturation and respiratory outcomes following exposure to fetal chronic hypoxaemia associated with IUGR in both clinical and animal studies (Gagnon *et al.* 1999; Braems *et al.* 2000; Cock *et al.* 2001; Orgeig *et al.* 2010; McGillick *et al.* 2016*c*). These different models support increased or decreased lung maturation, which parallels with either reduced or greater risk of respiratory complications in IUGR newborns in clinical practice (McGillick *et al.* 2016*c*). The different outcomes between studies may be due in part to the timing, severity and duration of the fetal hypoxaemic insult experienced in each model (Gagnon *et al.* 1999; Morrison, 2008; Orgeig *et al.* 2010). Hence, it is necessary to tease apart the relative contributions of specific factors, including chronic fetal hypoxaemia, on fetal lung development and their role in the incidence of respiratory complications in IUGR newborns. Herein, we comprehensively investigate the effect of chronic fetal hypoxaemia in a model of late gestation onset IUGR on molecular and structural development of the preterm fetal lung.

Methods

Ethical approval

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Cambridge Ethical Review Committee. Experiments were designed and reported with reference to the ARRIVE guidelines (Kilkenny *et al.* 2014). The authors have read, and the experiments comply with the policies and regulations of *The Journal of Physiology* (Grundy, 2015).

Surgery

At 100 $\pm$ 1 days gestation (term, ~145 days), 16 pregnant Welsh mountain ewes carrying singleton pregnancies as determined by ultrasound scan (Toshiba Medical Systems Europe, Zoetermeer, Netherlands) underwent a laparotomy, as previously described (Brain *et al.* 2015). Antibiotics (30 mg kg^{-1} I.M. procaine benzylpenicillin; Depocillin; Intervet UK Ltd, Milton Keynes, UK) and an analgesic agent (1.4 mg kg^{-1} S.C. carprofen; Rimadyl; Pfizer Let, Sandwich, UK) were administered to the ewe immediately before surgery. Briefly, general anaesthesia was induced by Alfaxan (1.5–2.5 mg kg^{-1}, I.V. administration, alfaxalone; Jurox Ltd, Worcestershire, UK) and maintained with 1.5–2.0% isofluorane (Datex-Ohmeda Ltd, Hatfield, UK). Following midline abdominal incision and uterotomy, the fetal hind limbs were exposed and the fetal sex was determined. To control for possible sex differences only males were included in this study (female fetuses were assigned to a postnatal study). Fetuses were returned to the intrauterine cavity and catheters were placed in the maternal femoral artery and maternal femoral vein as previously described (Brain *et al.* 2015). Following surgery, ewes were housed in individual floor pens with a 12:12 h light–dark cycle with free access to hay and water. From 100 days, ewes were fed daily with a maintenance diet to facilitate the monitoring of food intake (Brain *et al.* 2015).

Experimental protocol

At 103 days of gestation, ewes were randomly assigned to either of two experimental groups: normoxic ($n = 8$) or hypoxic (MCH, $n = 8$). Ewes allocated to the normoxic group remained housed in individual floor pens for the duration of the experimental protocol. Pregnant ewes assigned to the hypoxic group were housed in bespoke isobaric hypoxic chambers (Telstar Ace, Dewsbury, UK) from 103 days under normoxic conditions and exposed to ~10% O_2 from 105 days by altering the incoming inspirate mixture as described previously (Brain *et al.* 2015). Maternal feed intake was measured daily and was not different between the two groups (Allison *et al.* 2016*a*; Brain *et al.* 2015).

Arterial blood gas measurements

Maternal arterial blood samples were taken daily for measurement of blood gases (ABL5 blood gas analyser; Radiometer; Copenhagen, Denmark; measurements corrected to 38 °C) and percentage saturation of haemoglobin with oxygen (Sat Hb; measured using a haemoximeter; OSM3, Radiometer) (Brain *et al.* 2015).

Post-mortem and tissue collection

Fetuses were evaluated in late gestation (138 days; term = 145 days) when the lung is in the alveolar stage of development similar to human late preterm birth (36–37 weeks of gestation; term = 40 weeks). At 138 days of gestation, ewes in the hypoxic group were transferred from the hypoxic chambers to the post-mortem laboratory wearing a respiratory hood providing the same hypoxic gas mixture (Brain *et al.* 2015). All ewes and their fetuses were killed by an overdose of sodium pentobarbitone (0.4 ml kg^{-1}, i.v. administration, Pentoject; Animal Ltd, York, UK) and fetuses were delivered by hysterotomy. A fetal umbilical arterial blood sample was taken for measurement of haemoglobin (Hb; Radiometer; measurements corrected to 39.5 °C) and plasma cortisol concentration. Fetal body and organ weights, bi-parietal diameter and lower limb length were recorded. A piece of left fetal lung tissue was snap frozen in liquid nitrogen and stored at −80°C for lung tissue cortisol determination and gene expression analysis. A section of right fetal lung tissue was immersion fixed in 4% paraformaldehyde and processed to paraffin for further immunohistochemical analysis. Data from these animals characterising the maternal physiology and cardiovascular outcomes following exposure to MCH have been published previously (Allison *et al.* 2016*a*; Brain *et al.* 2015). As this cohort was not specifically run with lung analysis as a primary outcome, additional samples and analyses such as bronchoalveolar lavage and lung function measures were not available/possible.

Fetal plasma cortisol concentration

Plasma cortisol concentrations in fetal umbilical arterial blood collected at post-mortem (normoxic, $n = 8$; hypoxic, $n = 8$) were measured using a commercially available ELISA kit (RE52061, IBL International, Hamburg, Germany), according to the manufacturer's guidelines as previously described (Brain *et al.* 2015).

Fetal lung tissue cortisol concentration

Fetal lung tissue (∼50 mg; normoxic, $n = 7$; hypoxic, $n = 6$) was sonicated (Kinematica PT-MR-3100, Lucerne, Switzerland) in 250 μl of 1× PBS. Cortisol was extracted from the supernatant following addition of 100 μl extraction buffer (Oxford Biomedical Research, Rochester Hills, MI, USA) and 2 ml ethyl ether (VWR, Qld, Australia) and vortexed for 1 min followed by 5 min phase separation. The organic phase (1.2 ml) was dried at 37°C with air for 30 min and re-suspended with extraction buffer (120 μl). Neat test samples were assayed in addition to the standards on a Cortisol Enzyme Immunoassay according to the manufacturer's guidelines (EA65, Oxford Biomedical Research).

Quantification of mRNA transcripts within the fetal lung

For total RNA extraction, total RNA was extracted from all 16 lung tissue samples (∼50 mg; normoxic, $n = 8$; hypoxic, $n = 8$) using QIAzol Lysis Reagent Solution and Qiagen miRNeasy purification columns (Qiagen, Victoria, Australia) and cDNA was synthesised using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) as previously described (McGillick *et al.* 2015, 2016*b*).

For quantitative real-time RT-PCR (qRT-PCR) output normalisation three stable reference genes, beta-actin (*ACTB*), peptidylprolyl isomerase A (*PPIA*) (Passmore *et al.* 2009) and tyrosine 3-monooxygenase (*YWHAZ*) (McGillick *et al.* 2013) were chosen from a panel of candidate reference genes using the geNorm component of qbase^{plus} 2.0 software (Biogazelle, Zwijnaarde, Belgium) and run in parallel with target genes as described previously (McGillick *et al.* 2013). Expression of genes regulating oxidative stress, hypoxia signalling, glucocorticoid signalling, lung liquid movement, surfactant maturation and airway remodelling (Table 1) were measured by qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 6 μl on a ViiA7 Fast Real-time PCR system (Applied Biosystems) as described previously (McGillick *et al.* 2013, 2015, 2016*b*). The abundance of each transcript relative to the abundance of stable reference genes was calculated using DataAssist 3.0 analysis software and is expressed as mRNA mean normalised expression (MNE) ± SD (McGillick *et al.* 2013).

Quantification of SFTP-B-positive cells within the fetal lung by immunohistochemistry

Immunohistochemistry was performed (normoxic, $n = 6$; hypoxic, $n = 8$), using a rabbit anti-human mature surfactant protein B (SFTP-B) antibody (1:500, WRAB-48604, Seven Hills Bioreagents, Cincinnati, OH, USA) as previously described (Lock *et al.* 2015; McGillick *et al.*, 2015, 2016*b*). All sections were counterstained with Mayer's Haematoxylin (Sigma Aldrich, St Louis, MO, USA). Negative control slides were incubated overnight at 4 °C in parallel with test slides under the same experimental conditions as described previously (Lock *et al.* 2015; McGillick *et al.* 2015, 2016*b*). Stained sections were examined using Visiopharm new Computer Assisted Stereological Toolbox (NewCAST) software (Visiopharm, Hoersholm, Denmark) as described previously (McGillick *et al.* 2013, 2015, 2016*b*; Lock *et al.* 2015). Sixty counting

Table 1. Evaluation of target genes regulating oxidative stress, hypoxia signalling, glucocorticoid signalling, lung liquid movement (controlled by chloride, sodium and water movement), surfactant maturation and airway remodelling by quantitative real-time RT-PCR

Gene name	Gene symbol	Primer concentration (μM)	Primer reference
Pro-oxidant markers			
Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase	*NOX-4*	0.45	McGillick *et al.* (2016*b*)
Heme oxygenase-1	*HMOX-1*	0.90	McGillick *et al.* (2016*b*)
Inducible nitric oxide synthase	*NOS-2*	0.45	McGillick *et al.* (2016*b*)
Endothelial nitric oxide synthase	*NOS-3*	0.45	McGillick *et al.* (2016*b*)
Anti-oxidant markers			
Superoxide dismutase enzymes	*SOD-1*	0.45	McGillick *et al.* (2016*b*)
	SOD-2	0.45	
Catalase	*CAT*	0.90	McGillick *et al.* (2016*b*)
Glutathione peroxidase	*GPX*	0.45	McGillick *et al.* (2016*b*)
Hypoxia signalling			
Hypoxia inducible factor subunits	*HIF-1*α	0.45 (F), 0.90 (R)	Botting *et al.* (2014)
	HIF-2α	0.45	
	HIF-3α	0.45	
	HIF-1β	0.45	
Vascular endothelial growth factor	*VEGF*	0.45	Botting *et al.* (2014)
Adrenomedullin	*ADM*	0.45	Botting *et al.* (2014)
Lysine (K)-specific demethylase 3A	*KDM3A*	0.45	Botting *et al.* (2014)
Solute carrier family 2 (facilitated glucose transporter) member 1	*SLC2A1*	0.45	Botting *et al.* (2014)
Egl-9 family hypoxia-inducible factor enzymes (encoding the prolyl hydroxylase domain (PHD) proteins)	*EGLN-1* (PHD-2)	0.45	Botting *et al.* (2014)
	EGLN-2 (PHD-1)	0.45	
	EGLN-3 (PHD-3)	0.45	
Glucocorticoid signalling			
11β-hydroxysteroid dehydrogenase enzyme isoforms	*HSD11B-1*	0.90	McGillick *et al.* (2013)
	HSD11B-2	0.45	
Glucocorticoid receptor	*NR3C1*	0.90	McGillick *et al.* (2013)
Mineralocorticoid receptor	*NR3C2*	0.90	McGillick *et al.* (2013)
Chloride transport			
Cystic fibrosis transmembrane conductance regulator	*CFTR*	0.45	McGillick *et al.* (2016*b*)
Chloride channel voltage-sensitive 2 channel	*CLCN2*	0.45	McGillick *et al.* (2016*b*)
Sodium transport			
Epithelial sodium channel subunits	*SCNN1-A*	0.90	McGillick *et al.* (2013)
	SCNN1-B	0.45	
	SCNN1-G	0.45	
Sodium potassium adenosine triphosphatase subunits	*ATP1-A1*	0.45	McGillick *et al.* (2013)
	ATP1-B1	0.45	
Water transport			
Aquaporins	*AQP-1*	0.45	McGillick *et al.* (2013)
	AQP-3	0.45	
	AQP-4	0.45	
	AQP-5	0.45	
Surfactant maturation and lipid transport			
Surfactant proteins	*SFTP-A*	0.30	Orgeig *et al.* (2010)
	SFTP-B	0.30	McGillick *et al.* (2013)
	SFTP-C	0.30	
	SFTP-D	0.30	
Phosphate cytidylyltransferase 1, choline, alpha	*PCYT1A*	0.45	McGillick *et al.* (2015)
ATP-binding cassette, sub-family A (ABC1), member 3	*ABCA3*	0.45	McGillick *et al.* (2015)

(*Continued*)

Table 1. Continued

Gene name	Gene symbol	Primer concentration (μM)	Primer reference
Airway remodelling			
Elastin	*ELN*	0.90	McGillick *et al.* (2015)
Collagen type 1 alpha 1	*COL1A1*	0.45	McGillick *et al.* (2015)
Reference genes			
Beta-actin	*ACTB*	0.45	Passmore *et al.* (2009)
Peptidylprolyl isomerase A	*PPIA*	0.45 (F), 0.90 (R)	Passmore *et al.* (2009)
Tyrosine 3-monooxygenase	*YWHAZ*	0.45	McGillick *et al.* (2013)

Optimised final concentrations are the same for forward (F) and reverse (R) primers for target and reference genes unless otherwise indicated.

frames (600× magnification) of the alveolar epithelium were randomly selected per tissue section. Positive staining was confirmed with the presence of cuboidal shaped cells exhibiting cytoplasmic staining within the alveolar epithelium of lung tissue sections. Point-counting using an unbiased counting frame with an area of 20,000 μm^2 on immersion fixed tissue was used to estimate the numerical density of SFTP-B-positive cells within the alveolar epithelium of fetal lung tissue sections as described previously (McGillick *et al.* 2013; Lock *et al.* 2015).

Statistical analyses

Our specific research question relates to differences in outcome between the hypoxic (MCH) and normoxic groups in all measures and our data analysis has been carried out in consultation with a biostatistician. In the case of mRNA expression data, all genes were chosen *a priori* due to their known roles in normal development to prepare the lung for the successful transition to air-breathing at birth and have previously shown changes in response to altered intrauterine conditions (Walther *et al.* 1991; Flecknoe *et al.* 2003; Jesse *et al.* 2009; McGillick *et al.* 2013, 2016*b*). Therefore, we have chosen a statistically significant P value of ≤ 0.05 with no correction for multiple comparisons (Rothman, 1990; Greenland *et al.* 2016). All data are presented as mean ± SD or MNE ± SD in the case of gene expression data. Maternal blood gas values are presented as an average of the entire sampling period (105–138 days of gestation). Data were analysed using an effect size calculator (Excel) and adjusted P-values for mean difference (two-tailed t test) are presented in Table 2 in addition to the effect size and 95% confidence intervals for all measures.

Results

Impact of MCH on blood gas status of the ewe and fetus and fetal growth

MCH resulted in decreased partial pressure of oxygen (P_{aO_2}), partial pressure of carbon dioxide (P_{aCO_2}) and the saturation of haemoglobin with oxygen (Sat Hb), but increased haemoglobin content (Hb) in maternal arterial blood throughout the duration of the experimental protocol (Table 2). There was no effect of MCH on maternal pH (Table 2). Fetuses exposed to MCH had increased umbilical arterial Hb content at post-mortem (Table 2). Fetuses exposed to MCH had lower body weights, increased relative brain weight and elevations in the bi-parietal diameter to hind lower limb length ratio and brain to liver weight ratio, providing evidence of asymmetric IUGR compared with the normoxic group (Table 2). There was decreased total fetal lung weight, but no effect of MCH on relative fetal lung weight (Table 2).

Expression of genes regulating oxidative and nitrosative stress and anti-oxidant defence in the fetal lung

There was reduced mRNA expression of the pro-oxidant marker *NOX-4* (Fig. 1*A*) in the fetal lung following exposure to MCH. However, the levels of expression of an oxidative stress marker *HMOX-1* (Fig. 1*B*) and nitrosative stress regulators *NOS-2* (Fig. 1*C*) and *NOS-3* (Fig. 1*D*) were similar between groups. Furthermore, lung mRNA expression of the anti-oxidant *CAT* increased (Fig. 1*G*) following exposure to MCH, although there was no effect on expression of other anti-oxidant markers, including *SOD-1* (Fig. 1*E*), *SOD-2* (Fig. 1*F*) and *GPX* (Fig. 1*H*).

Expression of genes regulating hypoxia signalling and feedback in the fetal lung

There was increased fetal lung *HIF-3α* mRNA expression (Fig. 2*C*) following exposure to MCH, but no effect on gene expression of *HIF-1α* (Fig. 2*A*), *HIF-2α* (Fig. 2*B*) or *HIF-1β* (Fig. 2*D*) subunit expression. There was no effect of MCH on *VEGF* (Fig. 3*A*) or *ADM* (Fig. 3*B*) expression, but there was increased expression of hypoxia responsive genes *KDM3A* (Fig. 3*C*) and *SLC2A1* (Fig. 3*D*). *EGLN-3* mRNA expression increased (Fig. 4*C*), but there was no effect on *EGLN-1* (Fig. 4*A*) or *EGLN-2* (Fig. 4*B*), in the lung of fetuses following MCH.

Table 2. Analytical data demonstrating *P*-values for mean difference, effect size and 95% confidence intervals for the effect of maternal chronic hypoxia (MCH) compared to Controls on maternal and fetal characteristics, expression of genes regulating lung development, concentrations of cortisol in plasma and lung tissue and evaluation of surfactant producing cells in the fetal lung by immunohistochemistry

	Treatment group (Hypoxic)			Control group (Normoxic)								Confidence interval for difference					Confidence interval for effect size		
Outcome measure	Mean	*n*	SD	Mean	*n*	SD	Pooled SD	*P*-value for difference in SDs	Mean difference	*P*-value for mean diff (2-tailed *t* test)	Sediff	Lower	Upper	Effect size	Bias-corrected (Hedges)	Standard error of E.S. estimate	Lower	Upper	Effect size based on control group SD
Maternal Characteristics																			
P_{aO_2} (mmHg)	47	8	2.8	106	8	2.9	2.85	0.464	−59.00	<0.001	1.43	−61.79	−56.21	−20.70	−19.57	3.49	−44.03	4.90	−20.34
P_{aCO_2} (mmHg)	29.9	8	1.5	37.3	8	0.9	1.24	0.101	−7.40	<0.001	0.62	−8.61	−6.19	−5.98	−5.66	1.12	−13.48	2.17	−8.22
pH	7.5	8	0.016	7.49	8	0.006	0.012	0.010	0.01	0.132	0.006	−0.002	0.02	0.83	0.78	0.52	−2.85	4.41	1.67
Sat Hb (%)	80	8	3.28	103.6	8	1.5	2.55	0.028	−23.60	<0.001	1.28	−26.10	−21.10	−9.25	−8.75	1.63	−20.12	2.63	−15.73
Hb (g dl^{-1})	11.7	8	0.6	10	8	0.4	0.51	0.153	1.70	<0.001	0.26	1.20	2.20	3.33	3.15	0.75	−2.09	8.39	4.25
O_2 content	5.5	8	0.59	6.11	8	0.71	0.65	0.319	−0.61	0.083	0.33	−1.25	0.03	−0.93	−0.88	0.52	−4.55	2.78	−0.86
Fetal characteristics at post-mortem																			
Umbilical arterial Hb (g dl^{-1})	16.4	7	1.6	13.6	7	1.4	1.51	0.33	2.81	0.004	0.81	1.24	4.39	1.87	1.75	0.63	−2.65	6.15	2.07
Body weight (kg)	3.2	7	0.9	4.0	8	0.4	0.66	0.04	−0.84	0.047	0.36	−1.54	−0.14	−1.27	−1.20	0.56	−5.13	2.73	−2.08
Ratio of bi-parietal diameter to hind limb lower length	6.5	6	1.6	3.7	6	0.3	1.13	0.002	2.78	0.008	0.66	1.49	4.06	2.45	2.26	0.74	−2.91	7.43	8.67
Total brain weight (g)	42.0	7	6.5	43.4	8	2.8	4.889	0.03	−1.43	0.605	2.66	−6.64	3.78	−0.29	−0.28	0.52	−3.92	3.36	−0.51
Relative brain weight (g kg^{-1})	13.9	7	2.6	10.9	8	0.5	1.805	0.001	2.96	0.025	0.99	0.99	4.91	1.64	1.54	0.59	−2.58	5.66	5.55
Relative brain/liver weight ratio	0.7	7	0.2	0.5	8	0.0	0.114	0.002	0.25	0.005	0.06	0.13	0.38	2.21	2.08	0.64	−2.41	6.58	6.35
Total lung weight (g)	71.3	6	20.3	104.3	8	21.2	20.847	0.48	−33.03	0.013	11.18	−54.94	−11.11	−1.58	−1.48	0.61	−5.74	2.78	−1.56
Relative lung weight (g kg^{-1})	23.0	6	3.1	26.1	8	4.2	3.799	0.26	−3.06	0.145	1.96	−6.90	0.79	−0.80	−0.75	0.56	−4.66	3.16	−0.72
Pro-oxidant markers																			
NOX-4	0.0032	7	0.0009	0.0052	7	0.0016	0.001	0.094	−0.0020	0.018	0.0007	−0.003	−0.001	−1.54	−1.44	0.60	−5.64	2.76	−1.25
HMOX-1	0.0265	8	0.0059	0.0287	8	0.0103	0.008	0.082	−0.0022	0.611	0.0042	−0.010	0.006	−0.26	−0.25	0.50	−3.76	3.27	−0.21
NOS-2	0.0184	8	0.0054	0.0185	7	0.0024	0.004	0.026	−0.0001	0.963	0.0021	−0.004	0.004	−0.02	−0.02	0.52	−3.64	3.60	−0.04
NOS-3	0.0064	7	0.0011	0.0074	8	0.0015	0.001	0.234	−0.0010	0.162	0.0007	−0.002	0.000	−0.75	−0.71	0.53	−4.44	3.03	−0.67
Anti-oxidant markers																			
SOD-1	0.4009	8	0.0637	0.3874	7	0.0244	0.050	0.012	0.0135	0.593	0.0243	−0.034	0.061	0.27	0.26	0.52	−3.38	3.89	0.55
SOD-2	0.0420	8	0.0143	0.0364	8	0.0107	0.013	0.231	0.0056	0.391	0.0063	−0.007	0.018	0.44	0.42	0.51	−3.12	3.96	0.52
CAT	0.2071	8	0.0508	0.1583	8	0.0340	0.043	0.156	0.0488	0.043	0.0216	0.006	0.091	1.13	1.07	0.53	−2.67	4.81	1.44
GPX	0.0147	8	0.0059	0.0101	8	0.0045	0.005	0.246	0.0046	0.103	0.0026	−0.001	0.010	0.88	0.83	0.52	−2.82	4.48	1.02
Hypoxia signalling and feedback																			
HIF-1α	0.0366	7	0.0052	0.0376	8	0.0068	0.006	0.265	−0.0010	0.753	0.0031	−0.007	0.005	−0.16	−0.15	0.52	−3.78	3.47	−0.15
HIF-2α	0.0720	8	0.0411	0.0578	8	0.0206	0.033	0.044	0.0142	0.403	0.0163	−0.018	0.046	0.44	0.41	0.51	−3.12	3.95	0.69
HIF-3α	0.0417	8	0.0182	0.0219	8	0.0064	0.014	0.007	0.0198	0.018	0.0068	0.006	0.033	1.45	1.37	0.56	−2.52	5.26	3.09
HIF-1β	0.0385	8	0.0091	0.0355	8	0.0073	0.008	0.288	0.0030	0.480	0.0041	−0.005	0.011	0.36	0.34	0.50	−3.18	3.87	0.41
VEGF	0.1614	8	0.0603	0.1312	8	0.0278	0.047	0.029	0.0302	0.227	0.0235	−0.016	0.076	0.64	0.61	0.51	−2.97	4.19	1.09
ADM	0.0173	7	0.0025	0.0152	8	0.0021	0.002	0.343	0.0021	0.106	0.0012	0.000	0.004	0.92	0.86	0.54	−2.92	4.65	1.00
KDM3A	0.0610	7	0.0075	0.0532	8	0.0021	0.005	0.003	0.0078	0.032	0.0029	0.002	0.014	1.47	1.38	0.58	−2.65	5.41	3.71
SLC2A1	0.0220	8	0.0063	0.0114	8	0.0013	0.005	0.000	0.0106	0.002	0.0023	0.006	0.015	2.33	2.20	0.63	−2.23	6.64	8.15
EGLN-1	0.0666	8	0.0170	0.0617	8	0.0072	0.013	0.019	0.0049	0.472	0.0065	−0.008	0.018	0.38	0.35	0.50	−3.17	3.88	0.68
EGLN-2	0.0216	8	0.0031	0.0222	8	0.0040	0.004	0.259	−0.0006	0.743	0.0018	−0.004	0.003	−0.17	−0.16	0.50	−3.66	3.35	−0.15
EGLN-3	0.0253	7	0.0069	0.0090	8	0.0031	0.005	0.035	0.0163	0.0004	0.0028	0.011	0.022	3.13	2.94	0.75	−2.28	8.17	5.26

(*Continued*)

Table 2. Continued

Outcome measure	Treatment group (Hypoxic) Mean	*n*	SD	Control group (Normoxic) Mean	*n*	SD	Pooled SD	*P*-value for difference in SDs	Mean difference	*P*-value for mean diff (2-tailed *t* test)	Sediff	Confidence interval for difference Lower	Upper	Effect size	Bias-corrected (Hedges)	Standard error of E.S. estimate	Confidence interval for effect size Lower	Upper	Effect size based on control group SD
Glucocorticoid signalling																			
Fetal plasma cortisol (ng ml^{-1})	16.6	8	8.1	17.6	8	8.5	8.302	0.451	−1.0000	0.813	4.1512	−9.136	7.136	−0.12	−0.11	0.50	−3.62	3.39	−0.12
Lung tissue cortisol (pg mg^{-1})	4.8	6	2.4	4.1	7	1.6	2.010	0.174	0.7670	0.526	1.1580	−1.503	3.037	0.38	0.35	0.56	−3.57	4.28	0.49
HSD11B-1	0.0064	7	0.0024	0.0064	8	0.0024	0.002	0.500	0.0000	1.000	0.0013	−0.002	0.002	0.00	0.00	0.52	−3.62	3.62	0.00
HSD11B-2	0.0037	8	0.0012	0.0044	8	0.0009	0.001	0.233	−0.0007	0.210	0.0005	−0.002	0.000	−0.66	−0.62	0.51	−4.21	2.96	−0.78
NR3C1	0.1603	8	0.0252	0.1490	8	0.0214	0.023	0.339	0.0113	0.350	0.0117	−0.012	0.034	0.48	0.46	0.51	−3.09	4.00	0.53
NR3C2	0.0075	8	0.0022	0.0068	8	0.0020	0.002	0.404	0.0007	0.516	0.0011	−0.001	0.003	0.33	0.31	0.50	−3.21	3.84	0.35
Chloride transport																			
CFTR	0.0046	8	0.001	0.004	8	0.0005	0.001	0.044	0.0006	0.160	0.0004	0.000	0.001	0.76	0.72	0.52	−2.89	4.33	1.20
CLCN2	0.0036	8	0.0008	0.0034	8	0.0009	0.001	0.382	0.0002	0.646	0.0004	−0.001	0.001	0.23	0.22	0.50	−3.29	3.73	0.22
Sodium transport																			
SCNN1-A	0.0396	8	0.0111	0.0294	8	0.0113	0.011	0.482	0.0102	0.090	0.0056	−0.001	0.021	0.91	0.86	0.52	−2.80	4.52	0.90
SCNNI-B	0.0194	8	0.0044	0.0141	7	0.0042	0.004	0.447	0.0053	0.033	0.0022	0.001	0.010	1.23	1.16	0.56	−2.76	5.07	1.26
SCNN1-G	0.0085	8	0.0030	0.0078	8	0.0051	0.004	0.092	0.0007	0.744	0.0021	−0.003	0.005	0.17	0.16	0.50	−3.35	3.66	0.14
ATP1-A1	0.0462	8	0.0089	0.0337	8	0.0035	0.007	0.012	0.0125	0.005	0.0034	0.006	0.019	1.85	1.75	0.59	−2.37	5.86	3.57
ATP1-B1	0.0241	8	0.0058	0.0153	8	0.0039	0.005	0.158	0.0088	0.004	0.0025	0.004	0.014	1.78	1.68	0.58	−2.39	5.76	2.26
Water movement																			
AQP-1	0.3166	8	0.1263	0.2146	8	0.0401	0.094	0.004	0.1020	0.061	0.0469	0.010	0.194	1.09	1.03	0.53	−2.70	4.75	2.54
AQP-3	0.0021	7	0.0009	0.0013	7	0.0006	0.001	0.173	0.0008	0.079	0.0004	0.000	0.002	1.05	0.98	0.57	−2.98	4.94	1.33
AQP-4	0.0166	8	0.0067	0.0091	8	0.0048	0.006	0.199	0.0075	0.023	0.0029	0.002	0.013	1.29	1.22	0.54	−2.59	5.03	1.56
AQP-5	0.0359	8	0.0087	0.0311	8	0.0076	0.008	0.365	0.0048	0.259	0.0041	−0.003	0.013	0.59	0.56	0.51	−3.01	4.12	0.63
Surfactant maturation and lipid transport																			
SFTP-A	0.6379	8	0.4099	0.4339	8	0.1808	0.317	0.023	0.2040	0.227	0.1584	−0.106	0.514	0.64	0.61	0.51	−2.97	4.19	1.13
SFTP-B	1.2957	8	0.4446	0.8471	8	0.2156	0.349	0.038	0.4486	0.028	0.1747	0.106	0.791	1.28	1.21	0.54	−2.59	5.02	2.08
SFTP-C	5.3256	8	2.2333	3.4354	8	1.1282	1.769	0.046	1.8902	0.058	0.8846	0.156	3.624	1.07	1.01	0.53	−2.71	4.73	1.68
SFTP-D	0.0446	8	0.0130	0.0294	8	0.0105	0.012	0.293	0.0152	0.023	0.0059	0.004	0.027	1.29	1.22	0.54	−2.59	5.03	1.45
PCYT1A	0.0250	8	0.0039	0.0260	8	0.0045	0.004	0.358	−0.0010	0.642	0.0021	−0.005	0.003	−0.24	−0.22	0.50	−3.74	3.29	−0.22
ABCA3	0.0405	8	0.0121	0.0298	7	0.0065	0.010	0.064	0.0107	0.053	0.0049	0.001	0.020	1.08	1.02	0.55	−2.83	4.86	1.65
Numerical density of SFTP-B-positive cells	328.0	8	92.5	293.4	6	33.4	73.82	0.009	34.60	0.354	’35.41	−34.80	104.0	0.47	0.44	0.55	−3.39	4.26	1.04
Airway remodelling																			
ELN	0.8503	7	0.1376	0.8556	7	0.2253	0.187	0.128	−0.0053	0.959	0.0998	−0.201	0.190	−0.03	−0.03	0.53	−3.77	3.72	−0.02
COL1A1	1.8623	8	0.9092	1.4439	7	0.7295	0.831	0.288	0.4184	0.341	0.4235	−0.412	1.248	0.50	0.47	0.52	−3.20	4.15	0.57

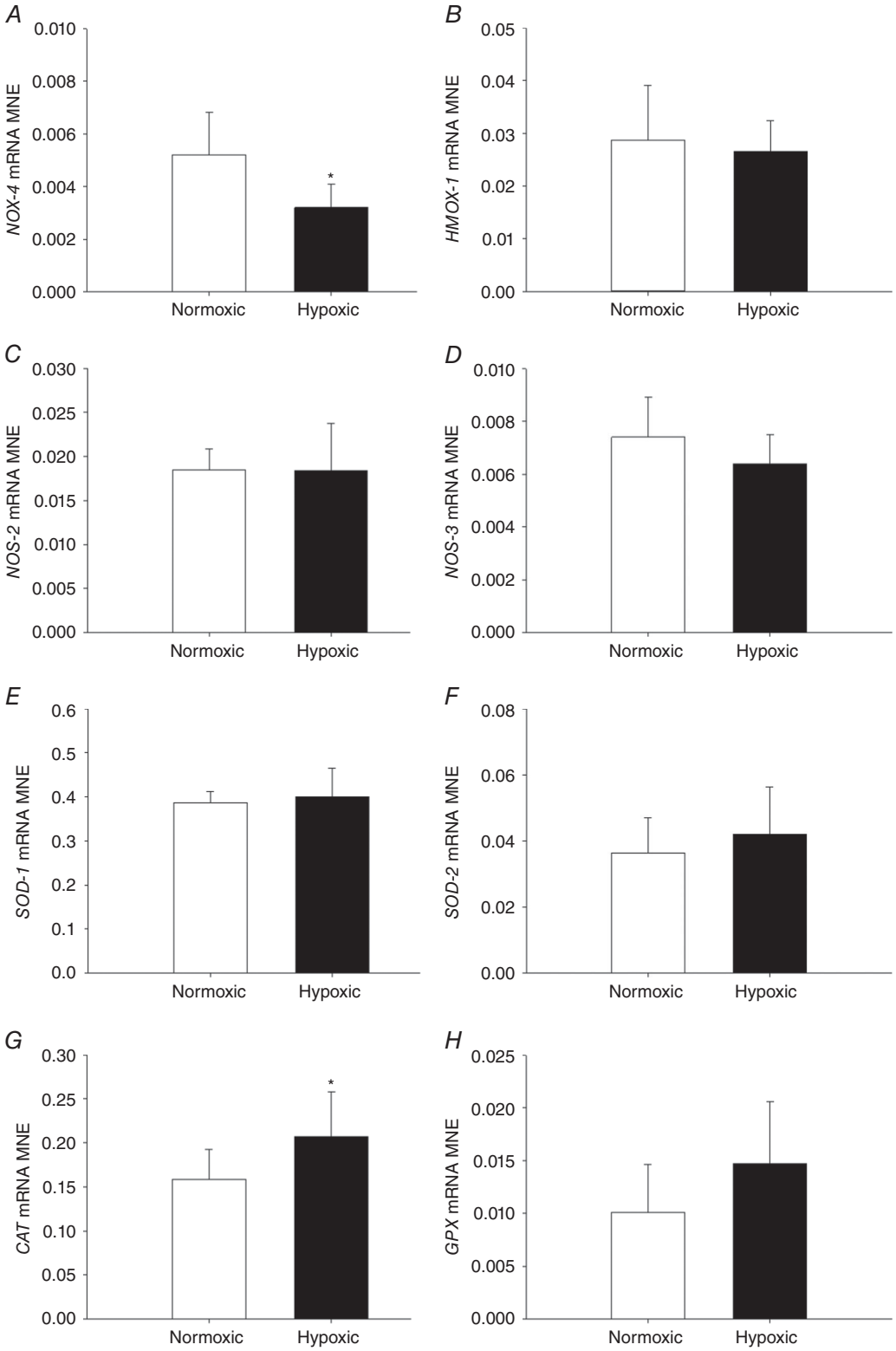

Figure 1. Maternal chronic hypoxia reduces expression of pro-oxidant marker *NOX-4* and increases expression of anti-oxidant marker *CAT*
Mean normalised expression (MNE) of genes regulating oxidative and nitrosative stress [*NOX-4* (*A*), *HMOX-1* (*B*), *NOS-2* (*C*) and *NOS-3* (*D*)] and anti-oxidant enzymes [*SOD-1* (*E*), *SOD-2* (*F*), *CAT* (*G*) and *GPX* (*H*)] in the lung of the late-gestation sheep fetus. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, P-value for mean difference between groups.

Fetal plasma cortisol, lung tissue cortisol and expression of genes regulating glucocorticoid signalling in the fetal lung

There was no effect of MCH on fetal plasma cortisol concentration (Fig. 5*A*) or total cortisol concentration in fetal lung tissue (Fig. 5*B*), when measured at 138 days of gestation. There was also no effect of MCH on fetal lung mRNA expression of genes regulating glucocorticoid (GC) availability (*HSD11B-1*, Fig. 5*C*; *HSD11B-2*, Fig. 5*D*) or activity (*NR3C1*, Fig. 5*E*; *NR3C2*, Fig. 5*F*) compared with the normoxic group.

Expression of genes regulating fetal lung liquid movement

MCH did not alter expression of genes regulating chloride movement (*CFTR*, Fig. 6*A*; *CLCN2*, Fig. 6*B*) in the fetal lung. There was a significant increase in expression of subunits regulating sodium movement, including *SCNN1-B* (Fig. 6*D*), *ATP1-A1* (Fig. 6*F*) and *ATP1-B1* (Fig. 6*G*) subunits, but no effect on *SCNN1-A* (Fig. 6*C*) or *SCNN1-G* (Fig. 6*E*) subunits, in the fetal lung following exposure to MCH. Exposure to MCH increased mRNA expression of *AQP-4* (Fig. 7*C*) in the fetal lung, but had no effect on *AQP-1* (Fig. 7*A*), *AQP-3* (Fig. 7*C*) or *AQP-5* expression (Fig. 7*D*).

Molecular and structural regulation of surfactant maturation and airway remodelling in the fetal lung

There was a significant increase in expression of *SFTP-B* (Fig. 8*B*) and *SFTP-D* (Fig. 8*D*), but not of *SFTP-A* (Fig. 8*A*) or *SFTP-C* (Fig. 8*C*), in the lung of fetuses exposed to MCH compared with the normoxic group. In addition, there was increased expression of the surfactant lipid transporter *ABCA3* (Fig. 8*F*) following exposure to MCH. There was no effect of MCH on expression of *PCYT1A* (Fig. 8*E*), a rate-limiting enzyme involved in surfactant phospholipid synthesis or genes regulating airway remodelling (*ELN*, Fig. 9*A*; *COL1A1*, Fig. 9*B*). There was no effect of MCH on the numerical density of SFTP-B-positive cells (Fig. 10*E*) present in the alveolar epithelium in the fetal lung tissue compared to the normoxic group.

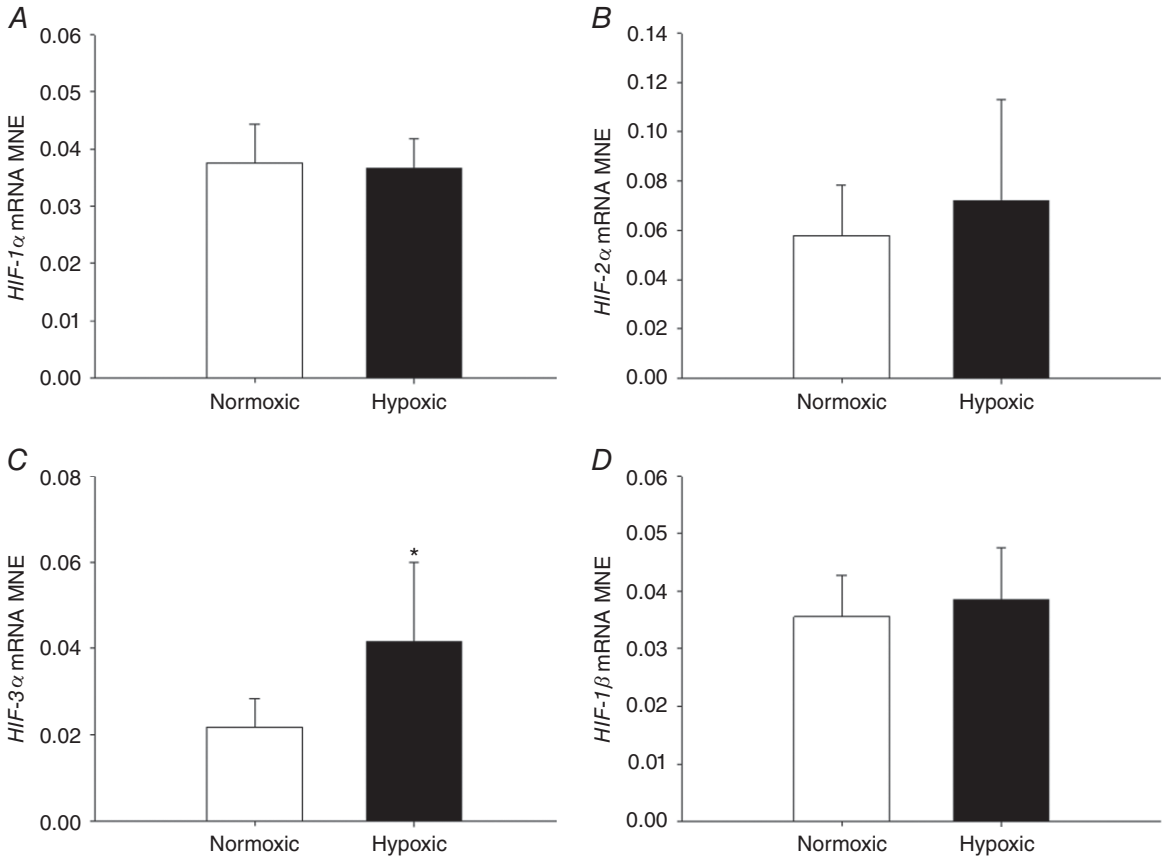

Figure 2. Maternal chronic hypoxia increases expression of genes regulating hypoxia signalling
Mean normalised expression (MNE) of genes regulating HIF signalling [*HIF-1α* (*A*), *HIF-2α* (*B*), *HIF-3α* (*C*) and *HIF-1β* (*D*)] in the lung of the late-gestation sheep fetus. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

Discussion

We have used isobaric hypoxic chambers that are able to maintain unrestrained pregnant ewes to isolate the effect of exposure to MCH for a month in late gestation on fetal lung maturation (Brain *et al.* 2015). Exposure to MCH without alteration in maternal food intake promoted increased expression of genes regulating hypoxia signalling, lung liquid reabsorption and surfactant maturation in the fetal lung (Fig. 11). The mechanisms underlying these effects appear to be regulated at the molecular level as there was no difference in the numerical density of SFTP-B-positive cells present in the alveolar epithelium. The molecular mechanisms mediating maturational effects of MCH on the fetal lung include alterations to hypoxia but not GC signalling as there was no difference in expression of genes regulating GC signalling or changes to maternal or fetal plasma cortisol concentration, nor elevations in fetal lung tissue cortisol concentration. Combined, the data provide evidence for an adaptive response of the fetal lung to prenatal chronic hypoxaemia to increase the expression of factors essential for lung maturation and the successful transition to the air-breathing environment at birth.

In most sheep models of IUGR, fetal arterial oxygenation is reduced by ∼5–8 mmHg compared to controls (Morrison, 2008; Herrera *et al.* 2016). In this sheep model of maternal chronic hypoxia, the change is ∼9–10 mmHg (Brain *et al.* 2015), akin to those measured in severe IUGR in humans (Hecher *et al.* 1995). In this model, the level of maternal hypoxia produced decreased mean fetal descending aortic P_{O_2} values to 11.5 ± 0.6 mmHg relative to a mean of 20.9 ± 0.5 mmHg in control fetuses of a normoxic pregnancy (Brain *et al.* 2015). The increased maternal and fetal arterial Hb content represents an established compensatory mechanism to increase the oxygen carrying capacity to tissues in response to MCH. Despite this, exposure to MCH induced significant asymmetric fetal growth restriction, as evidenced by a reduction in fetal body weight, and increases in the ratio of the bi-parietal diameter to lower limb length and in the brain to liver weight ratio, all accepted indices of persistent fetal brain sparing (Giussani, 2016).

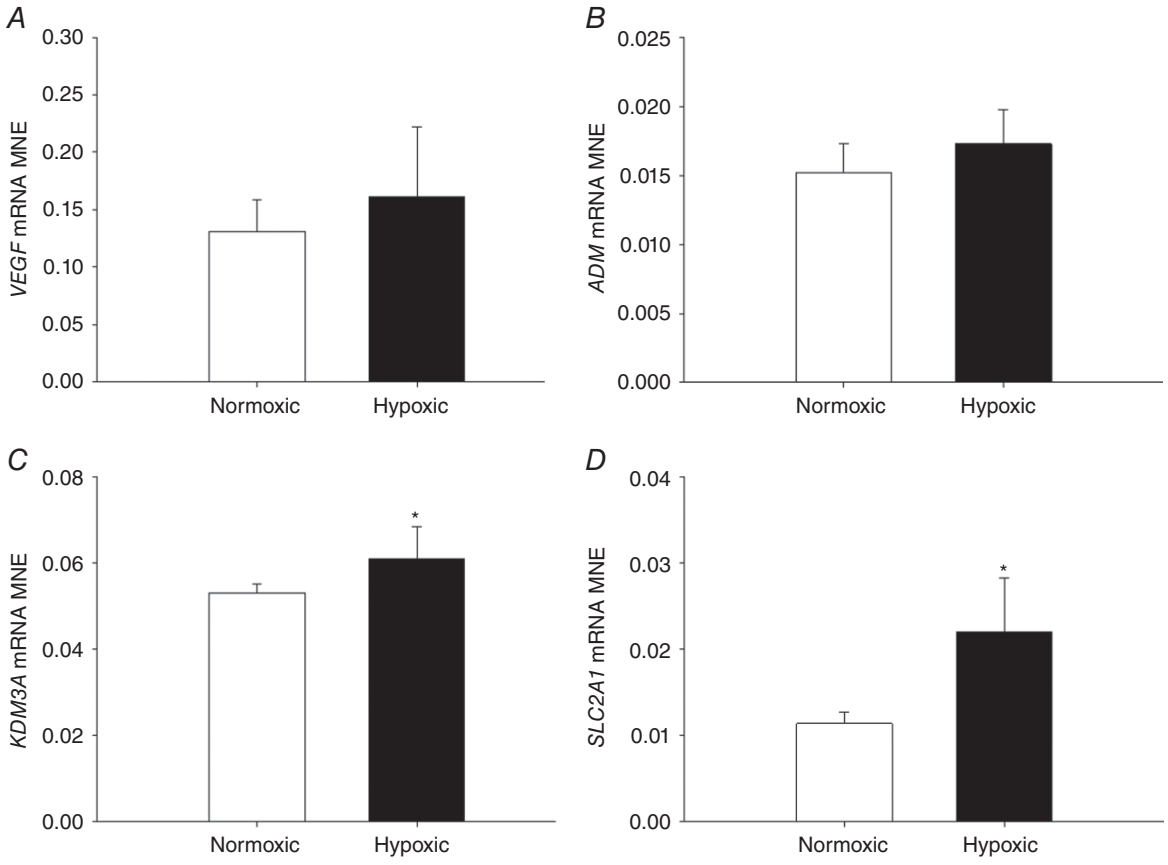

Figure 3. Maternal chronic hypoxia increases expression of hypoxia responsive genes in the fetal lung
Mean normalised expression (MNE) of genes with hypoxia response elements [*VEGF* (*A*), *ADM* (*B*), *KDM3A* (*C*) and *SLC2A1* (*D*)] in the fetal lung following exposure to maternal chronic hypoxia for a month in late gestation. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

An advantage of this sheep model over rodent models of IUGR in adverse pregnancy (Van Geijn *et al.* 1980) is that it isolates the contribution of chronic hypoxaemia on fetal growth and development independent of changes in maternal food intake or elevations in circulating maternal stress hormones, despite significant maternal hypoxaemia (Brain *et al.* 2015). This is important as maternal nutrient restriction and hypoxia can have differential effects on fetal organ development and these effects persist into postnatal life (Williams *et al.* 2005; Camm *et al.* 2010). Furthermore, in the present model of MCH, it is unlikely that the observed downstream changes in gene expression in the fetal lung at the time of tissue collection are GC dependent. This is advantageous as alterations in fetal oxygenation and circulating GCs can have differential effects on indices of lung maturation, as evidenced in other sheep models of IUGR resulting in fetal hypoxaemia (Gagnon *et al.* 1999; Orgeig *et al.* 2010, 2015; Allison *et al.* 2016*b*). Thus, despite the multi-factorial regulation of organ development by changes in metabolic and endocrine markers in the IUGR pregnancy, a strength of this study is the capacity to isolate the effect of fetal reduced oxygen availability to gain further understanding of the effect of chronic hypoxaemia on the molecular mechanisms regulating fetal lung development in adverse pregnancy.

In this study, only male fetuses were used to control for possible sex differences, which may be viewed as a potential limitation of our experimental design. However, it is important to note that evaluation of data from previous publications in our laboratory has demonstrated no effect of sex on expression of surfactant protein markers in the fetal lung at 133 days of gestation (Orgeig *et al.* 2015). While there is evidence to support a male respiratory disadvantage at birth, our previous findings in the PR early onset IUGR sheep model suggest that the effects observed between male and female fetuses would be similar in both the Control and the IUGR group in the current study.

Glucocorticoid signalling is essential for normal fetal and lung development and this is exploited clinically with antenatal GC administration to women at risk of preterm delivery to promote fetal lung maturation to ensure the successful transition to newborn life (McGillick *et al.* 2013; Antenatal Corticosteroid Clinical Practice Guidelines Panel, 2015). In this study, we have observed no effect of MCH on fetal plasma cortisol, lung tissue

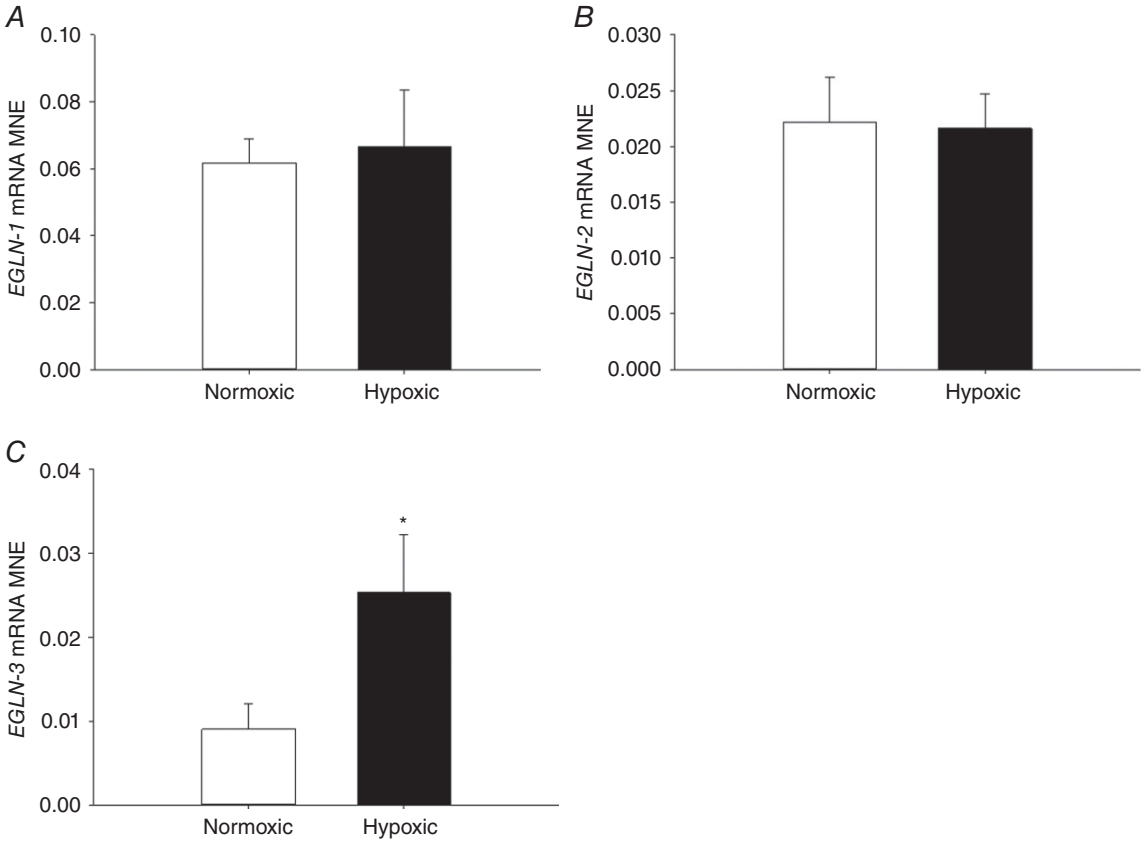

Figure 4. Maternal chronic hypoxia increases expression of hypoxia signalling regulatory factor *EGLN-3*
Mean normalised expression (MNE) of genes regulating hypoxia signalling and feedback [*EGLN-1* (*A*), *EGLN-2* (*B*) and *EGLN-3* (*C*)] in the lung of the late-gestation sheep fetus. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

cortisol concentration, or expression of genes regulating GC availability and activity at the time of tissue collection. The apparent limited effect on GCs in this model is consistent with other sheep models of chronic hypoxia, including exposure to long-term hypoxaemia induced by living at high altitude from mid- to late gestation (Harvey *et al.* 1993). However, this finding is in contrast to the chronically hypoxaemic PR fetus where there are persistent elevations in circulating GCs and altered expression of factors regulating GC availability and activity throughout gestation (Phillips *et al.* 1996; Orgeig *et al.* 2010, 2015). However, it is important to note that investigations in this study were undertaken on plasma and tissue samples collected at post-mortem. Therefore, it is possible that during the hypoxic insult for a month in late gestation, altered regulation of GC signalling at earlier time points

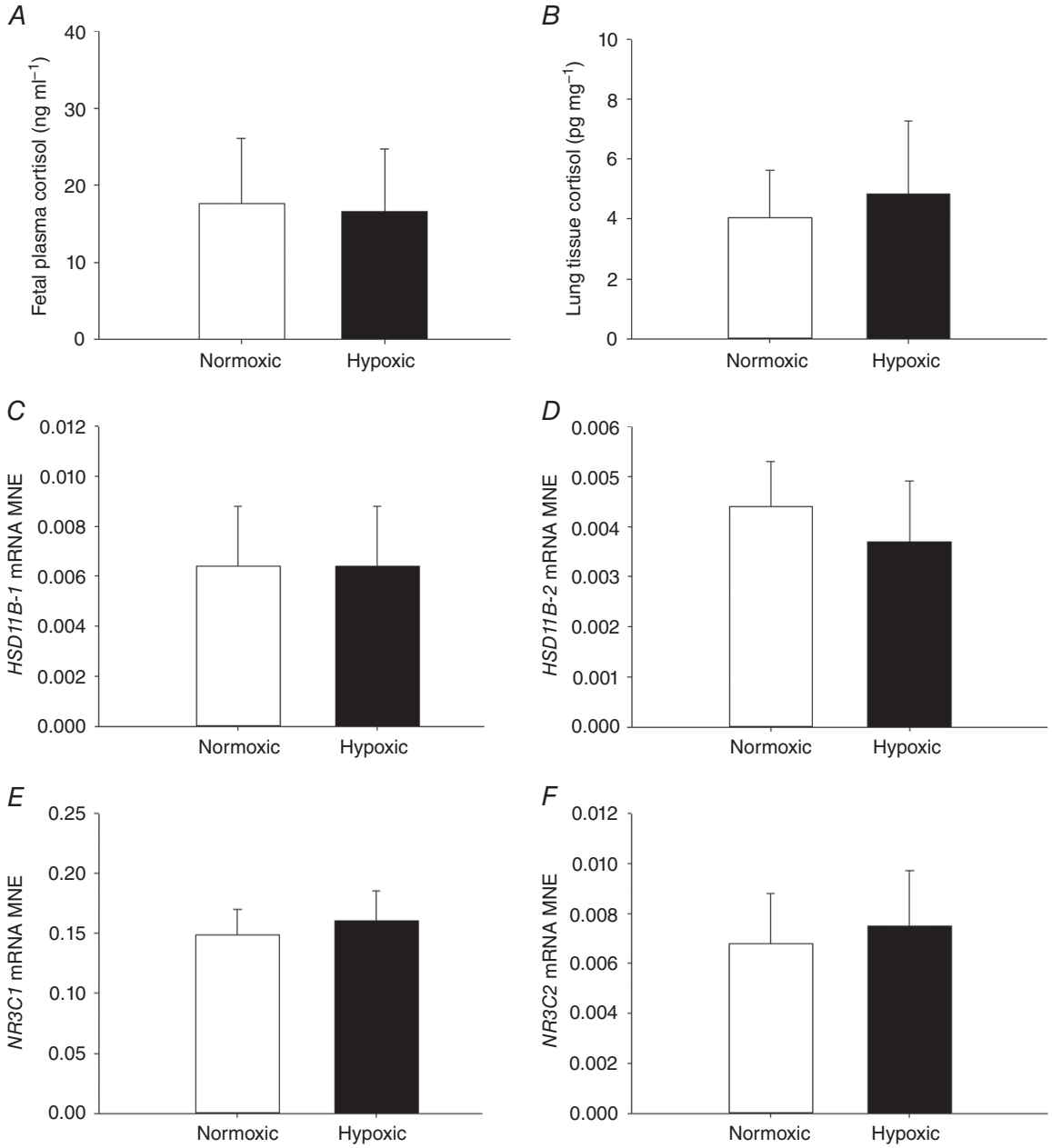

Figure 5. No effect of maternal chronic hypoxia on fetal glucocorticoid avaliability or signalling
Fetal plasma cortisol (*A*), total lung tissue cortisol (*B*) and mean normalised expression (MNE) of genes regulating glucocorticoid availability [*HSD11B-1* (*C*) and *HSD11B-2* (*D*)] and activity [*NR3C1* (*E*) and *NR3C2* (*F*)] in the fetal lung. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

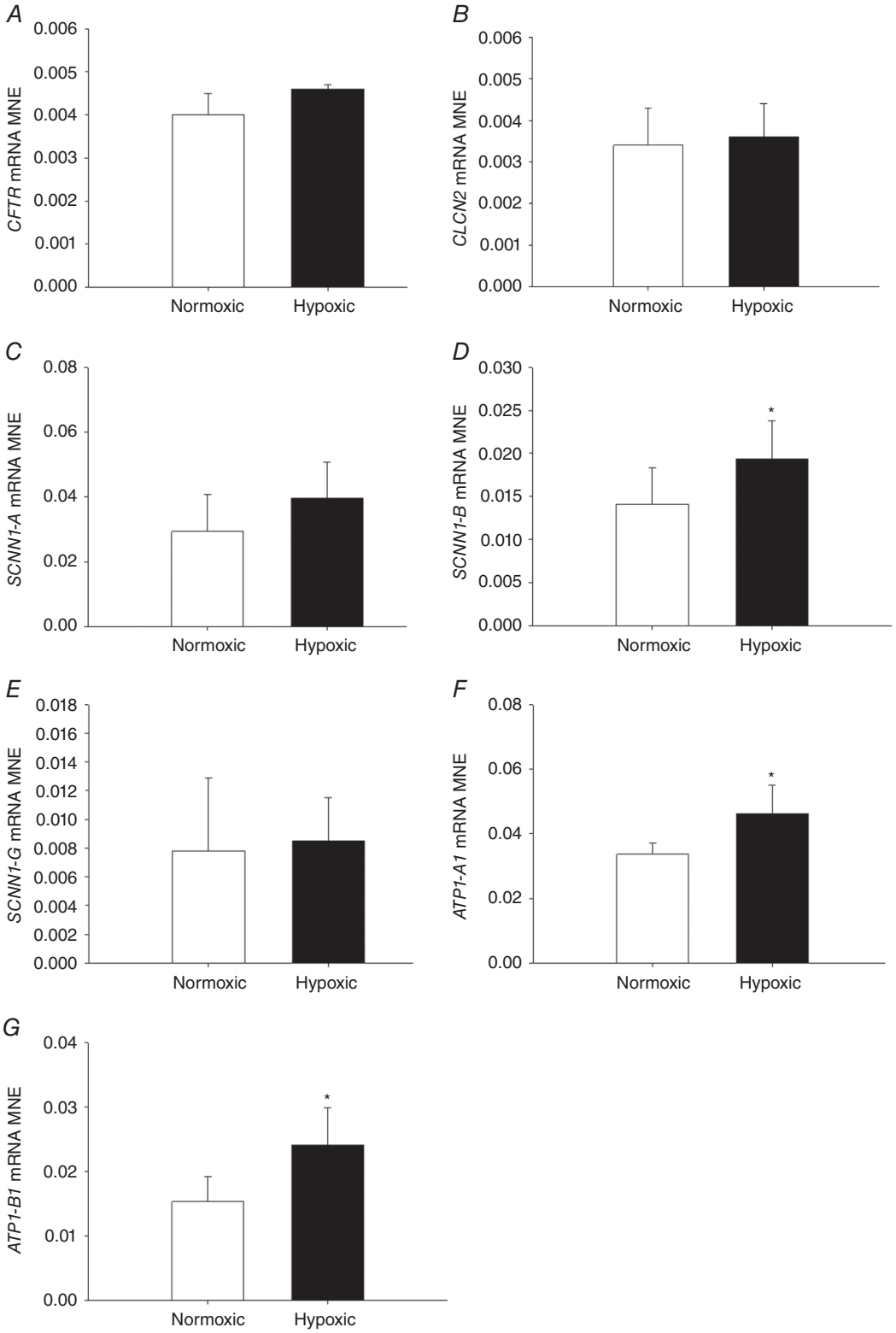

Figure 6. Maternal chronic hypoxia increases expression of genes regulating sodium movement in the fetal lung
Mean normalised expression (MNE) of genes regulating chloride [*CFTR* (*A*) and *CLCN2* (*B*)] and sodium [*SCNN1-A* (*C*), *SCNN1-B* (*D*), *SCNN1-G* (*E*), *ATP1-A1* (*F*) and *ATP1-B1* (*G*)] movement in the lung of the late-gestation sheep fetus. Data expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

following initiation of the hypoxic insult may have contributed to the overall changes observed in the fetal lung. Importantly, we have demonstrated that there is no difference in maternal catecholamines (Brain *et al.* 2015) and plasma cortisol concentration between the normoxic and hypoxic pregnancies in this model (104–138 days of gestation; 94.4 ± 16.8 *vs.* 74.5 ± 13.7 ng ml^{-1}; $P = 0.7$). Further characterisation of the model in the future will elucidate the specific mechanisms contributing to both maternal and fetal adaptations to MCH.

HIF-3α is regarded as a sensitive regulator of the tissue response to systemic hypoxia (Heidbreder *et al.* 2003). Increased *HIF-3α* expression has been observed following exposure to a 50% reduction in oxygen tension for 0.5–2 h, with no impact on *HIF-1α*, *HIF-2α* or *HIF-1β* transcription (Heidbreder *et al.* 2003). The present study confirms increased expression of *HIF-3α* in the fetal lung following exposure to MCH for a month in late gestation. Increased HIF-α signalling is further evidenced by increased expression of downstream genes with hypoxia response elements, including *SLC2A1* and *KDM3A*. An advantage of measuring expression of genes with hypoxia response elements in their promoter region is that they are under direct control of HIF-α subunit stability and hence act as a more functional outcome than HIF-α protein expression itself. The increased expression of *KDM3A*, which encodes the histone demethylase, jumonji domain containing 1A (JMJD1A), suggests an interesting potential role of MCH by epigenetic modifications to the regulation of fetal lung maturation at the transcriptional level (Wellmann *et al.* 2008). In this model, MCH also increased *EGLN-3* expression, which encodes PHD-3 in the fetal lung. This finding is consistent with altered pulmonary regulation of hypoxia signalling by increased *EGLN*/PHD expression, as has been reported for the lung (Orgeig *et al.* 2015) and heart (Botting *et al.* 2014) of the chronically hypoxaemic fetus in late gestation as a result of placental restriction.

Increased hypoxia signalling following exposure to MCH occurred together with positive downstream effects on molecular regulation of lung liquid movement and surfactant maturation. Positive effects of hypoxia on both biochemical and physiological determinants of lung liquid movement have been reported previously (Vivona *et al.* 2001; Dada *et al.* 2003; Abreu-Rodríguez *et al.* 2011). However, there is limited *in vivo* evidence for the effect of

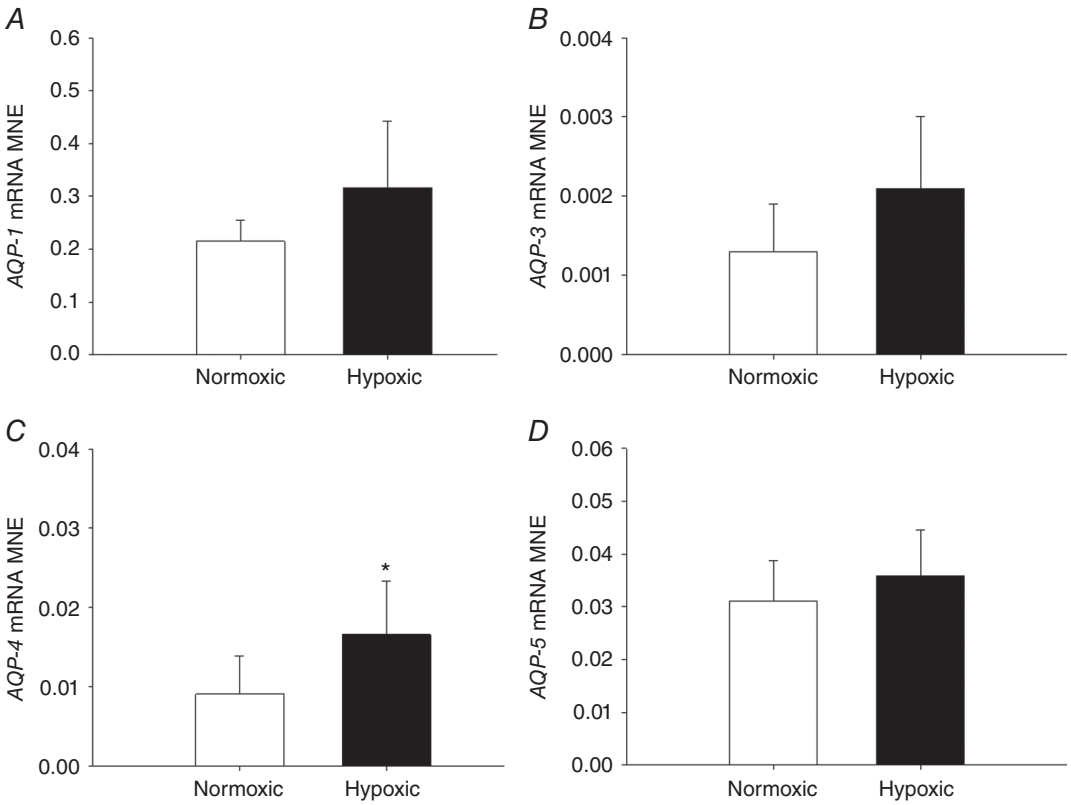

Figure 7. Maternal chronic hypoxia increases expression of gene regulating water movement in the fetal lung
Mean normalised expression (MNE) of genes regulating water movement [*AQP-1* (*A*), *AQP-3* (*B*), *AQP-4* (*C*) and *AQP-5* (*D*)] in the lung of the late-gestation sheep fetus. Data expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. *$P < 0.05$, *P*-value for mean difference between groups.

chronic hypoxaemia on molecular mechanisms regulating fetal lung liquid movement. In addition, the timing, duration and severity of the hypoxic insults may have a differential impact on the magnitude of change in these factors (Vivona *et al.* 2001; Dada *et al.* 2003; Morrison, 2008; Abreu-Rodríguez *et al.* 2011). For instance, we have previously reported that in the chronically hypoxaemic PR fetus, where the insult occurs prior to conception and growth restriction begins early in gestation, there are few effects of chronic hypoxaemia and IUGR on the molecular mechanisms regulating lung liquid movement (McGillick *et al.* 2016*b*). In contrast, here, following exposure to MCH for a month in late gestation we show a significant increase in the expression of genes regulating sodium (*SCNN1-B*, *ATP1-A1* and *ATP1-B1*) and water (*AQP-4*) movement in fetal lung tissue. These molecular changes may contribute in part to the control of lung liquid reabsorption in preparation for air-breathing at birth and play a functional

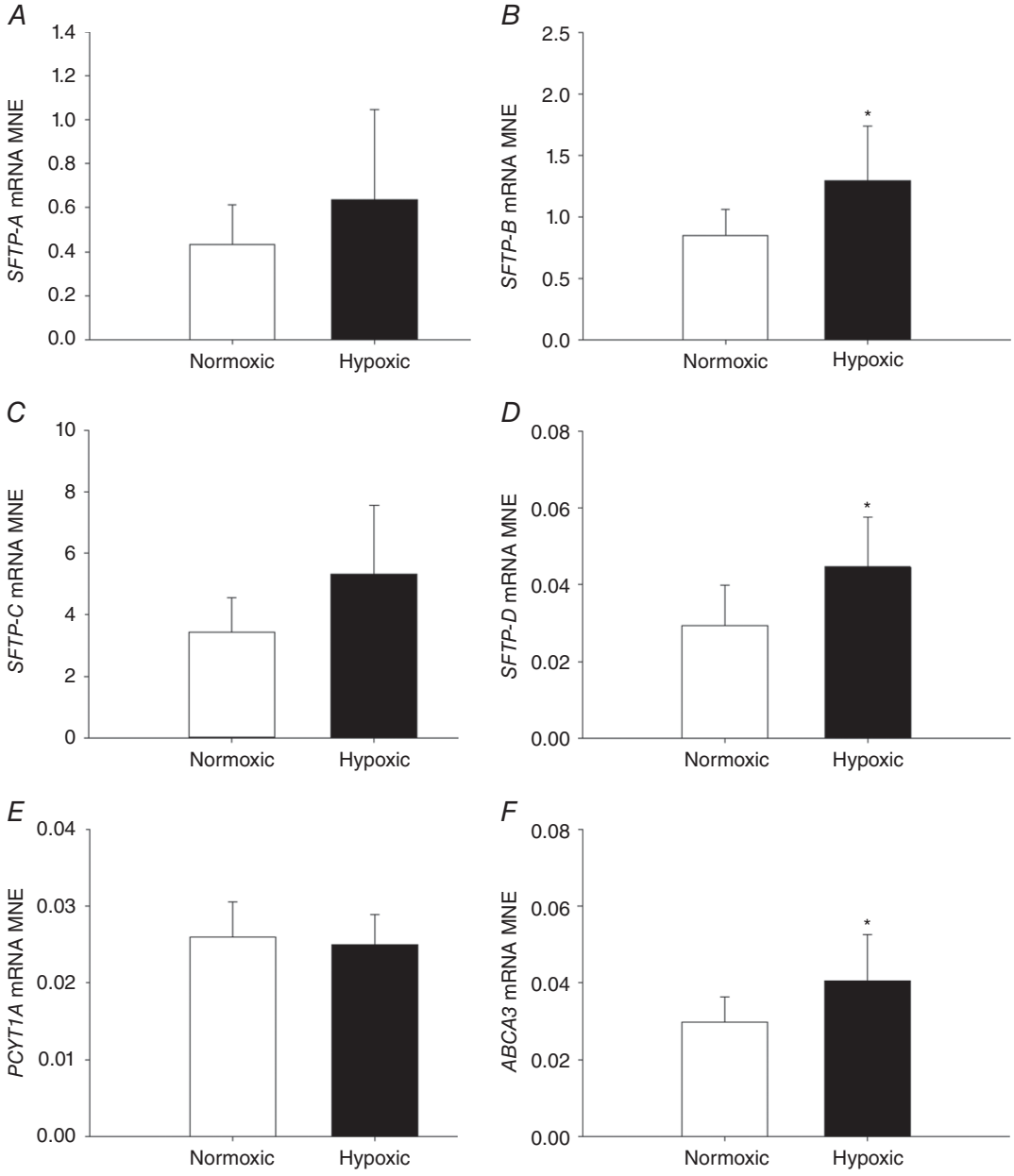

Figure 8. Maternal chronic hypoxia increases expression of genes regulating surfactant maturation and surfactant lipid transport in the fetal lung
Mean normalised expression (MNE) of genes regulating surfactant maturation [*SFTP-A* (*A*), *SFTP-B* (*B*), *SFTP-C* (*C*), *SFTP-D* (*D*)] and surfactant lipid synthesis and transport [*PCYT1A* (*E*) and *ABCA3* (*F*)] in the lung of the late-gestation sheep fetus. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. *$P \leq 0.05$, *P*-value for mean difference between groups.

role in regulation of lung liquid movement at the air–liquid interface in the neonatal lung (Hooper *et al.* 2015).

We also provide mechanistic molecular evidence for upregulation of the surfactant system with increased expression of *SFTP-B* and *SFTP-D* and the surfactant lipid transporter *ABCA3* in the fetal lung following MCH. Interestingly, our data suggest that exposure to MCH regulates surfactant maturation at the molecular level by increasing the functional capacity of surfactant-producing cells rather than by an overall change to the numerical density of SFTP-B-positive cells in fetal lung tissue as determined by immunohistochemistry. Evaluation of fetal lung tissue at the cellular level by immunohistochemistry is advantageous as it provides specific detail regarding functional capability of cells able to produce surfactant in contrast to ultrastructural detail of alveolar epithelial cell differentiation achieved with electron microscopy (Lock *et al.* 2015). The finding of increased molecular regulation of surfactant maturation in this model of MCH is consistent with other sheep models of fetal hypoxaemia, including utero-placental embolisation (Gagnon *et al.* 1999) or short-term 48 h of maternal inhalational hypoxia in late gestation (Braems *et al.* 2000). However, these present and past data contrast with findings in the chronically hypoxaemic PR fetus, in which there is reduced SFTP expression in the lung (Orgeig *et al.* 2010; McGillick 2015 and 2016*b*). Therefore, it is clear that surfactant maturation in the fetal lung can be differentially regulated by chronic hypoxaemia of varying duration, severity and/or time of onset. Moreover, these findings provide molecular evidence for the impact of chronic hypoxaemia on the heterogeneity observed in respiratory distress syndrome in IUGR neonates at birth (McGillick *et al.* 2016*c*).

While we were unable to measure the incidence of fetal breathing movements as the fetuses in this study were not catheterised, in our previous work in chronically hypoxaemic PR fetuses there was no effect on the incidence, amplitude or frequency of breathing movements in late gestation (Poudel *et al.* 2015; McGillick *et al.* 2016*b*). This study supports the notion of chronic hypoxaemia as a molecular regulator of fetal lung development. In contrast, a previous study showed reduced breathing movements measured by diaphragmatic electromyogram across late gestation in a group of hypoxaemic and acidotic growth-restricted fetuses that did not have brain sparing (Maloney *et al.* 1982). This parallels with human clinical literature with acidotic and hypoxic fetuses exhibiting reduced breathing movements (Yoneyama *et al.* 1994). As the fetuses in this model of MCH are not acidotic (Allison *et al.* 2016*a*) and exhibit characteristic growth restriction including brain sparing, it is likely that there would be no effect of this model on fetal breathing movements.

Finally, despite no change in the expression of *NOS-2* and *NOS-3* (key regulators of free radical generation contributing to nitrosative stress) or of *HMOX-1* (a molecular marker of oxidative stress), there was reduced expression of the pro-oxidant marker *NOX-4* and a significant increase in the anti-oxidant *CAT* in the fetal lung following exposure to MCH. These data suggest a compensatory shift in the upregulation of anti-oxidant pathways to offset oxidative stress and maintain homeostasis in the fetal lung. This may be an additional adaptation to protect the fetal lung from the relative hyperoxia following birth and one that appears to be regulated by hypoxia rather than GC signalling in this model of MCH.

The findings from this study highlight the complex regulation of lung development by chronic hypoxaemia associated with IUGR that supports the spectrum of respiratory distress syndrome outcomes that these infants may experience at birth (McGillick *et al.* 2016*c*). Despite providing mechanistic evidence for chronic hypoxaemia as

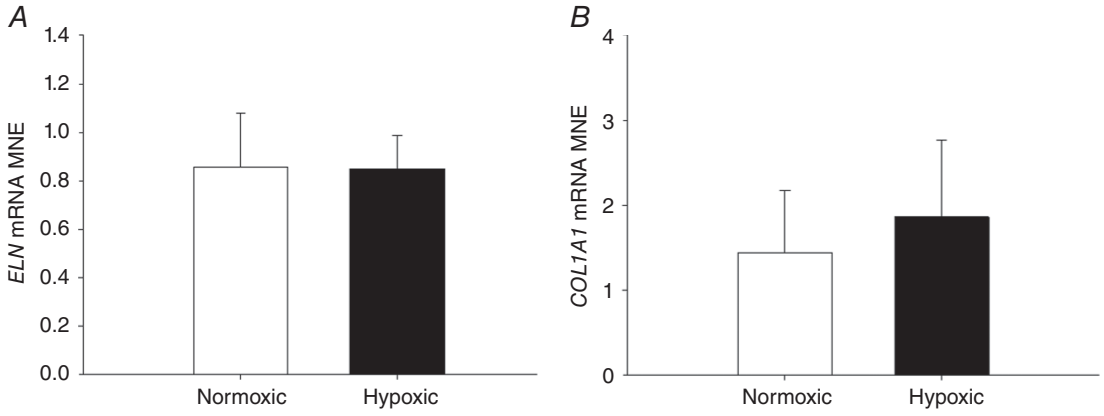

Figure 9. No effect of maternal chronic hypoxia on expression of genes regulating airway remodelling
Mean normalised expression (MNE) of *ELN* (*A*) and *COL1A1* (*B*) in the lung of the late-gestation sheep fetus. Data are expressed as mRNA MNE ± SD in normoxic (white bars) and hypoxic (black bars) pregnancies. $^*P < 0.05$, *P*-value for mean difference between groups.

a direct molecular regulator of key pathways contributing to fetal lung development, it is important to consider the possibility that overall the effects observed in this model of MCH may also be regulated synergistically or indirectly by a secondary mediator. With regards to alternative pathways that are potential regulators, we have previously investigated markers of cellular proliferation, beta-adrenergic receptors, interleukin 1-beta and transforming growth factor-beta following chronic fetal hypoxaemia in a PR model induced by uterine carunclectomy and found that these were not affected by chronic hypoxaemia (McGillick *et al.* 2015; Orgeig *et al.*

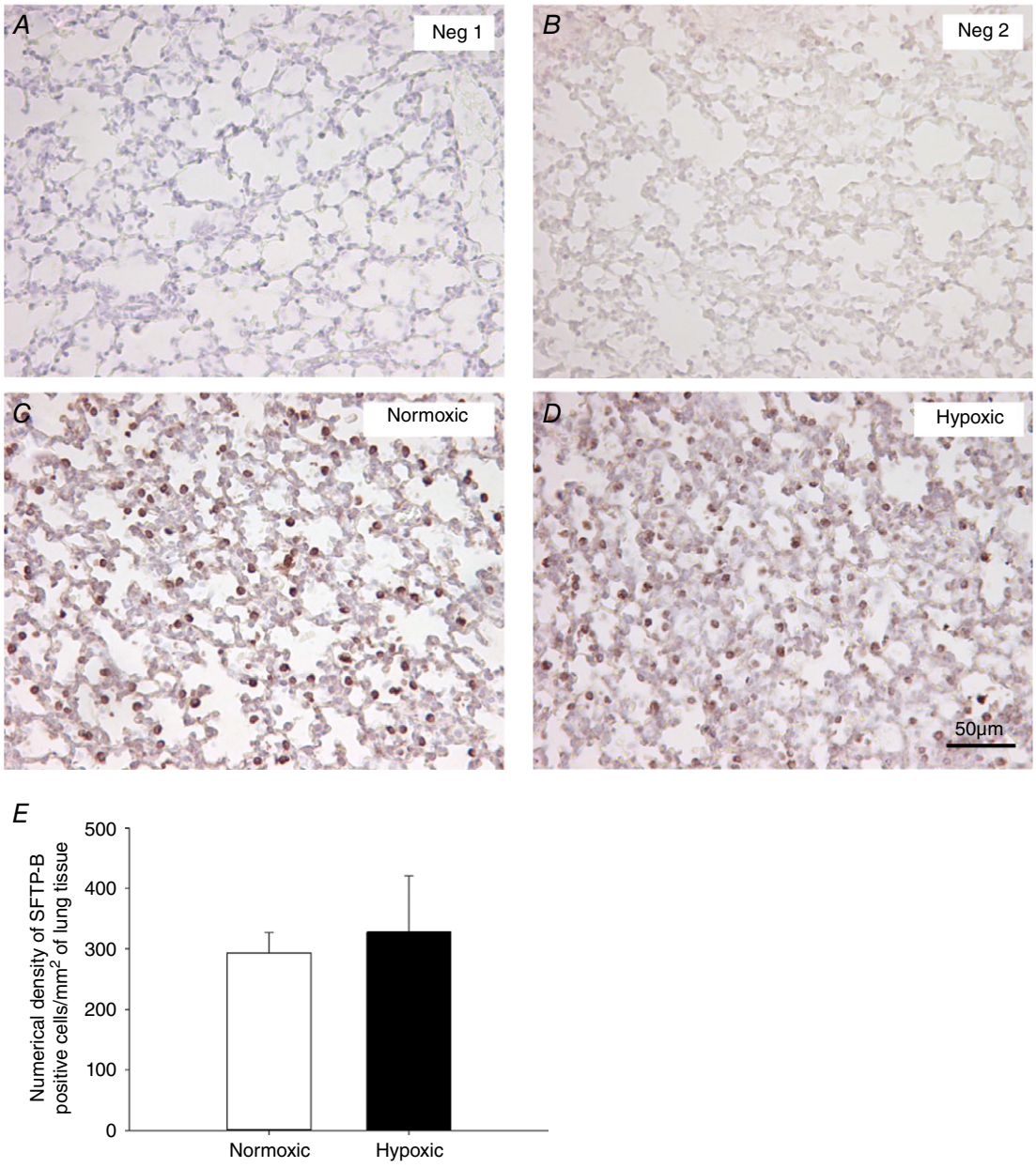

Figure 10. No effect of maternal chronic hypoxia on the numerical density of SFTP-B-positive cells in the alveolar epithelium of the fetal lung
Micrographs demonstrating no primary antibody-negative control (Neg 1, *A*), 1:500 rabbit serum negative control (Neg 2, *B*), SFTP-B immunoreactivity (brown intracellular precipitate) in the alveolar epithelium of the fetal lung tissue from normoxic (*C*) and hypoxic (*D*) pregnancies. There was no significant effect of maternal chronic hypoxia (black bar, *E*) on the numerical density of SFTP-B-positive cells per mm^2 of lung tissue in the alveolar epithelium when compared to lung tissue of fetuses from normoxic (white bar, *E*) pregnancies. Data are expressed as mean ± SD. $^*P < 0.05$, *P*-value for mean difference between groups. Micrographs at 200× magnification. Scale bar = 50 μm.

2015). Importantly, in this study we have investigated markers of oxidative stress, a process that represents an additional factor that may be potentially altered as a result of the relationship between hypoxia signalling and the generation of reactive oxygen species. Further characterisation of this MCH model in the future will elucidate additional mechanisms influencing fetal organ growth and development in response to the chronically hypoxaemic conditions experienced for a month in late gestation. In late gestation, studies in the PR sheep fetus showed a similar effect of chronic hypoxaemia on molecular regulators of lung maturation at both 133 and 140 days of gestation (Orgeig *et al.* 2010). These findings suggest that there is a similar response of the lung to chronic hypoxaemia throughout late gestation and, thus, it is reasonable to postulate that if the markers of lung maturation were evaluated closer to term (e.g. 145 days of gestation) there would be similar effects observed in the lung of the fetus following exposure to MCH in this model.

Importantly, this model of chronic hypoxaemia results in cardiovascular deficits similar to the human IUGR fetus (Allison *et al.* 2016*a*; Brain *et al.* 2015). These include fetal cardiovascular dysfunction, characterised by impaired myocardial contractility and diastolic function

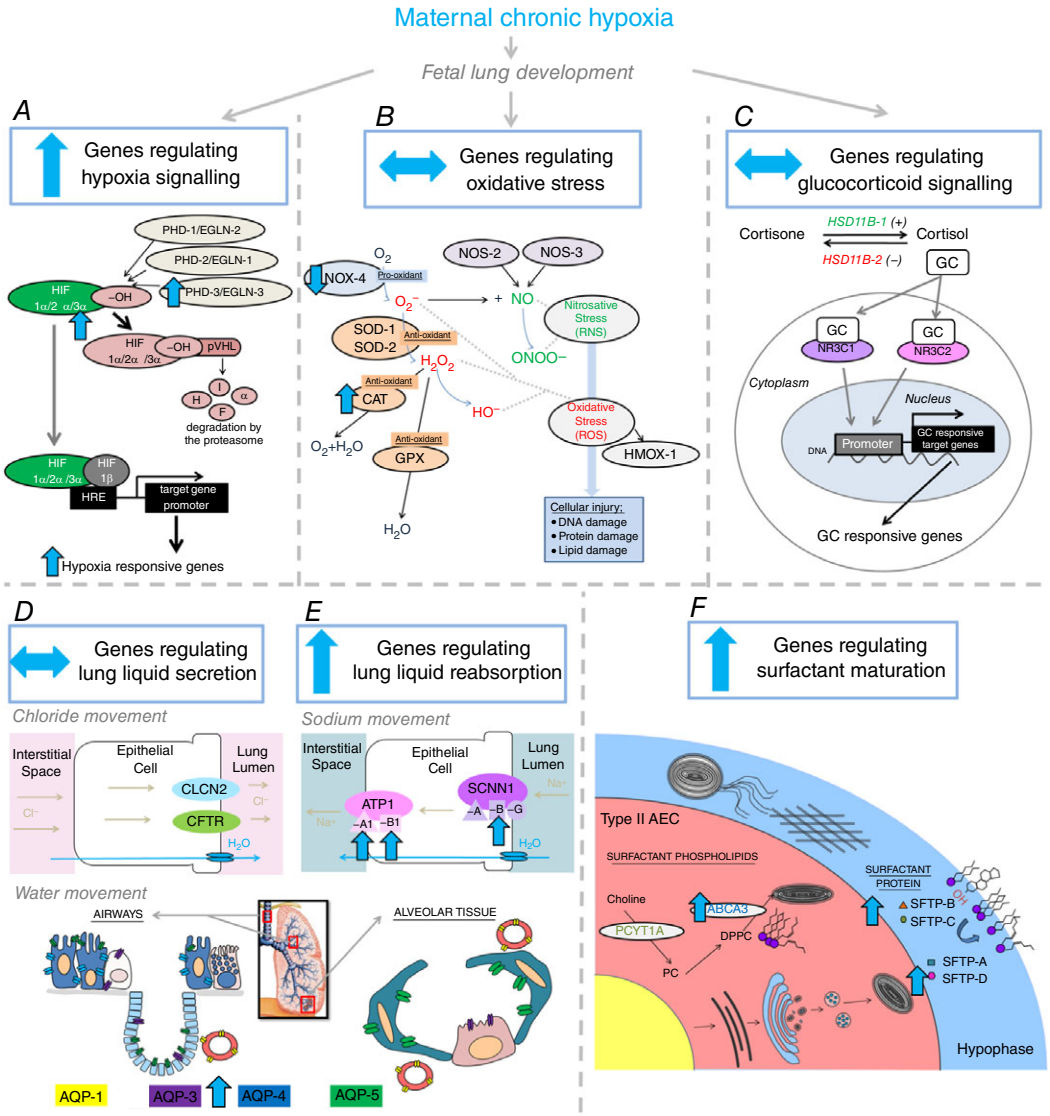

Figure 11. Overview of study findings on molecular signalling in the fetal lung
Effect of maternal chronic hypoxia for a month in late gestation on expression of genes regulating hypoxia signalling (*A*), oxidative stress (*B*), glucocorticoid signalling (*C*), lung liquid secretion (chloride movement; *D*), lung liquid reabsorption (sodium movement; *E*) and surfactant maturation (*F*) in the fetal lung. Overall maternal chronic hypoxia increased expression of genes regulating hypoxia signalling (*A*), lung liquid reabsorption (*E*) and surfactant maturation (*F*) in the fetal lung, which may be an adaptive response to aid in the successful transition to the air-breathing environment at birth.

as well as impaired peripheral vascular reactivity and loss of nitric oxide-dependent endothelial function (Brain *et al.* 2015). Interestingly, despite the negative cardiovascular effects of exposure to MCH, the fetus still has the capacity to enhance protective effects such as lung maturation to ensure successful transition to extrauterine life. These findings are consistent with studies showing decreased risk of respiratory distress syndrome in IUGR subpopulations (McGillick *et al.* 2016*c*), although this is in contrast to findings in the PR sheep fetus, which support increased risk of respiratory distress syndrome in a subset of IUGR newborns. Combined with our previous findings in a model of early-onset chronic hypoxaemia and IUGR (Orgeig *et al.* 2010, 2015; McGillick *et al.* 2015, 2016*b*), our work in this area highlights a differential effect of IUGR complicated by chronic hypoxaemia on fetal lung development that may underlie the heterogeneity in clinical newborn respiratory outcomes (McGillick *et al.* 2016*a*).

In summary, the data in the present study provide evidence for upregulation of the molecular mechanisms involved in processes vital for lung liquid movement and surfactant production in response to MCH (Fig. 11). This may be an adaptive response anticipating preterm birth triggered by adverse conditions experienced late in gestation. Overall, the study data suggest an increased functional capacity of the lung to ensure the adequate transition to air-breathing following exposure to late-gestation onset IUGR induced by MCH for a month in late gestation, thus reducing the risk of respiratory distress at birth and pulmonary dysfunction in later life.

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Additional information

Competing interests

The authors have no conflicts of interest.

Author contributions

EVM, SO, DAG and JLM were responsible for the conception and design of the experiments. EVM, SO, BJA, KLB, YN, NI, KLS, ADK, EAH, DAG and JLM were each involved in data acquisition. EVM, SO, DAG and JLM were involved in analysis and interpretation of the data. EVM, SO, DAG and JLM drafted the article. EVM, SO, BJA, KLB, YN, NI, KLS, ADK, EAH, DAG and JLM contributed to and have approved the final version to be published.

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Translational perspective

In this study we investigated the effect of maternal chronic hypoxia for a month in late gestation on molecular and structural regulation of fetal lung development. We isolated the effect of chronic hypoxaemia on fetal development using isobaric hypoxic chambers without alterations to maternal food intake. We have provided evidence that maternal chronic hypoxia in late gestation increases the expression of genes regulating hypoxia signalling, lung liquid reabsorption and surfactant maturation in the fetal lung. There was no effect of maternal chronic hypoxia on airway remodelling or structural fetal lung development. Therefore, we provide evidence for chronic fetal hypoxaemia as a molecular regulator of lung maturation. In contrast to other models of intrauterine growth restriction including early-onset chronic fetal hypoxaemia leading to reduced fetal lung maturation, late-onset fetal chronic hypoxaemia promotes molecular regulation of fetal lung development which may be an adaptive response in preparation for the successful transition to air-breathing at birth. Overall the findings from this study provide evidence for a differential effect of timing and duration of fetal chronic hypoxaemia on expression of factors regulating fetal lung maturation. This provides evidence for the heterogeneity observed in respiratory outcomes in newborns at birth following exposure to chronic hypoxaemia *in utero*. Greater understanding of the intrauterine environment encountered in growth-restricted pregnancies may lead to identification of newborns at altered risk of respiratory complications at birth in clinical practice.