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Glucocorticoids regulate mitochondrial fatty acid oxidation in fetal cardiomyocytes

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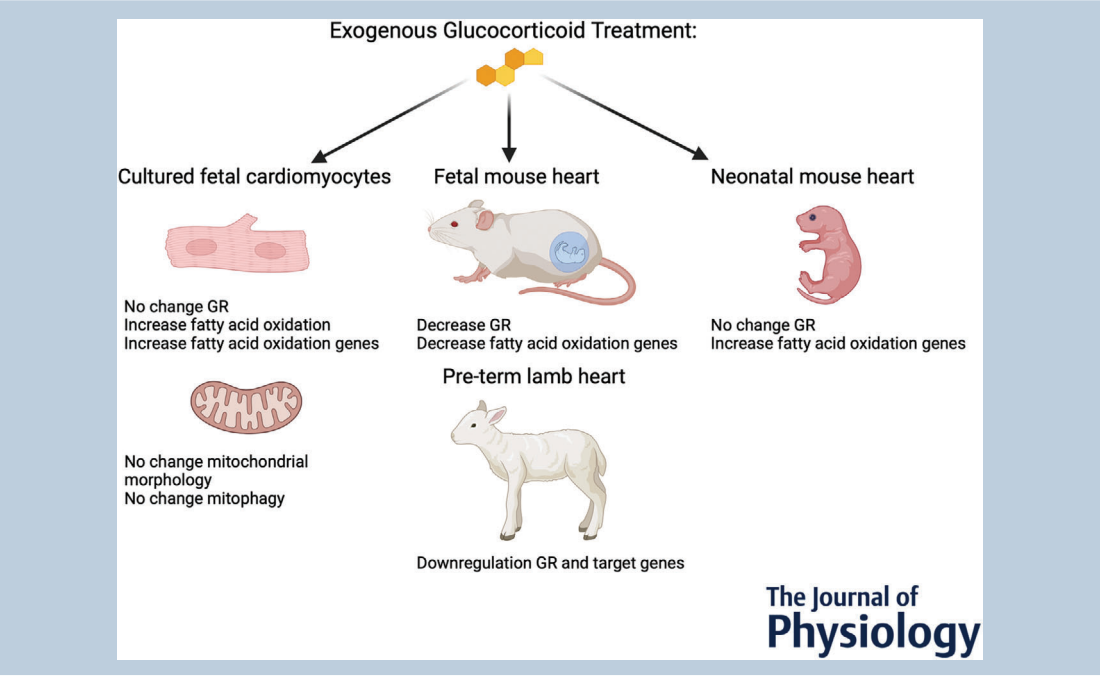

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Abstract The late gestational rise in glucocorticoids contributes to the structural and functional maturation of the perinatal heart. Here, we hypothesized that glucocorticoid action contributes to the metabolic switch in perinatal cardiomyocytes from carbohydrate to fatty acid oxidation. In primary mouse fetal cardiomyocytes, dexamethasone treatment induced expression of genes involved in fatty acid oxidation and increased mitochondrial oxidation of palmitate, dependent upon a glucocorticoid receptor (GR). Dexamethasone did not, however, induce mitophagy or alter the morphology of the mitochondrial network. *In vivo*, in neonatal mice, dexamethasone treatment induced cardiac expression of fatty acid oxidation genes. However, dexamethasone treatment of pregnant C57Bl/6 mice at embryonic day (E)13.5 or E16.5 failed to induce fatty acid oxidation genes in fetal hearts assessed 24 h later. Instead, at E17.5, fatty acid oxidation genes were downregulated by dexamethasone, as was GR itself. PGC-1α, required for glucocorticoid-induced maturation of primary mouse fetal cardiomyocytes *in vitro*, was also downregulated in fetal hearts at E17.5, 24 h after dexamethasone administration. Similarly, following a course of antenatal corticosteroids in a translational sheep model of preterm birth, both GR and PGC-1α were downregulated in heart. These data suggest that endogenous glucocorticoids support the perinatal switch to fatty acid oxidation in cardiomyocytes through changes in gene expression rather than gross changes in mitochondrial volume or mitochondrial turnover. Moreover, our data suggest that treatment with exogenous glucocorticoids may interfere with normal fetal heart maturation, possibly by down-regulating GR. This has implications for clinical use of antenatal corticosteroids when preterm birth is considered a possibility.

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Abstract figure legend Glucocorticoid treatment regulates fatty acid oxidation in cardiomyocytes. 24 hours after glucocorticoid treatment, capacity for mitochondrial fatty acid oxidation is increased in fetal cardiomyocytes in vitro (left) and in hearts of neonatal mice (right), with no alteration in expression of glucocorticoid receptor (GR). The increase in fatty acid oxidation in fetal cardiomyocytes in vitro occurs without any change in mitochondrial number or morphology (lower left). In contrast, fatty acid oxidation is down-regulated in fetal mice 24h after glucocorticoid treatment (centre), likely because of down-regulation of GR. GR is also down-regulated in preterm lambs following antenatal corticosteroid treatment in a sheep model of preterm birth. These findings suggest that antenatal glucocorticoid treatment may interfere with the normal trajectory of heart maturation by down-regulating GR.

Key points

- Glucocorticoids are steroid hormones that play a vital role in late pregnancy in maturing fetal organs, including the heart.
- In fetal cardiomyocytes in culture, glucocorticoids promote mitochondrial fatty acid oxidation, suggesting they facilitate the perinatal switch from carbohydrates to fatty acids as the predominant energy substrate.
- Administration of a synthetic glucocorticoid in late pregnancy in mice downregulates the glucocorticoid receptor and interferes with the normal increase in genes involved in fatty acid metabolism in the heart.
- In a sheep model of preterm birth, antenatal corticosteroids (synthetic glucocorticoid) down-regulates the glucocorticoid receptor and the gene encoding PGC-1α, a master regulator of energy metabolism.
- These experiments suggest that administration of antenatal corticosteroids in anticipation of preterm delivery may interfere with fetal heart maturation by downregulating the ability to respond to glucocorticoids.

Introduction

With birth and independent life comes the switch to breathing air and exposure to a pro-oxidant environment as well as a big increase in the workload of the heart and a substantial increase in energy demand. The dramatic increase in fetal glucocorticoid hormone concentration in late gestation is essential to support this transition from intrauterine to extrauterine life (Hillman *et al.* 2012; Rog-Zielinska *et al.* 2014). Administration of potent synthetic corticosteroids (betamethasone or dexamethasone) to pregnant women at risk of preterm delivery is standard care in high- and middle-income countries, with the aim of maturing the fetus to reduce neonatal morbidity and mortality (Kemp *et al.* 2016; Agnew *et al.* 2018). In addition to the well-known effects on lung maturation (Cole *et al.* 1995; Bird *et al.* 2015; Laresgoiti *et al.* 2016), glucocorticoids promote pro-survival adaptions in neonatal energy metabolism and in the cardiovascular system (Hillman *et al.* 2012). The heart undergoes profound changes at birth, with a marked increase in cardiac output and transition from a feto-placental blood circulation system to an independent circulatory system. In the perinatal period, cardiomyocyte proliferation ceases and cardiomyocytes undergo terminal differentiation, involving extensive binucleation and maturation of the myofibril structure, calcium handling and inter-cellular communication. Pre-clinical experiments, especially in sheep and rodent models, have demonstrated the impact of glucocorticoids and other hormones upon cardiac maturation (Hillman *et al.* 2012; Rog-Zielinska *et al.* 2014). However, which of the maturational effects of glucocorticoids on neonatal energy metabolism and in the cardiovascular system are directly attributable to glucocorticoid activation of the glucocorticoid receptor (GR) within tissues and which are mediated by other factors, such as thyroid hormones (Forhead & Fowden, 2014) remains uncertain. Indeed, there is a complex interdependence between glucocorticoid and thyroid hormones in maturation of organs and tissues in preparation for birth, with glucocorticoids being important for maturation of the thyroid axis (Hillman *et al.* 2012; Forhead & Fowden, 2014). Also unclear is whether antenatal administration of potent synthetic glucocorticoids like dexamethasone and betamethasone mimics endogenous glucocorticoid action in the fetal cardiovascular system. Indeed, exogenous glucocorticoid administration during late gestation 'programmes' an increased risk of cardiovascular disease in adulthood (Fowden *et al.* 1998; Rog-Zielinska *et al.* 2014). However, the direct effects of exogenous glucocorticoid administration on cardiomyocytes and other cells in the fetal heart (endothelial cells, fibroblasts, immune cells, etc.) remain unclear (reviewed, Rog-Zielinska *et al.* 2014; Agnew *et al.* 2018; Song *et al.* 2019). Our previous data suggest antenatal dexamethasone treatment dysregulates cardiac function and downregulates endogenous glucocorticoid action in the fetal heart (Agnew *et al.* 2019), potentially altering the normal trajectory of perinatal cardiac maturation.

The normal increase in fetal glucocorticoids in late gestation supports neonatal blood pressure (Hillman *et al.* 2012) and is essential to structurally and functionally mature the fetal heart (Thornburg *et al.* 2011; Rog-Zielinska *et al.* 2013). *In utero*, our 'SMGRKO' mice, with *Sm22-Cre*-mediated GR deficiency in cardiomyocytes and vascular smooth muscle cells show impaired heart function, disrupted cardiac ultrastructure and failure to induce key genes required for cardiac contractile function, calcium handling and energy metabolism (Rog-Zielinska *et al.* 2013). Supporting direct effects of GR, glucocorticoid treatment of primary mouse fetal cardiomyocytes *in vitro* matures ultrastructure and increases contractile function, mitochondrial capacity (O_2 consumption rate, basally and after uncoupling of mitochondria) and markers of cardiomyocyte maturation (Rog-Zielinska *et al.* 2015). Similarly, in human embryonic stem cell (ESC)-derived cardiomyocytes treated with dexamethasone, contractile force is increased and systolic calcium transient decay is faster (Kosmidis *et al.* 2015).

During the transition to a higher oxygen environment and a greater cardiac workload at birth, the cardiac preference for energy substrate switches. Substrates for mitochondrial oxidative phosphorylation derive from the tricarboxylic acid (TCA) cycle and other intermediary pathways. Reducing equivalents, NADH and $FADH_2$, generated through the TCA cycle are the source

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of electrons which transfer through complexes I to IV of the electron transport chain creating a proton gradient across the inner mitochondrial membrane. Electrons are ultimately transferred to O_2 to produce water or can be diverted to form superoxide and reactive oxygen species (ROS). ATP synthase (complex V) couples mitochondrial re-entry of protons to the generation of ATP from ADP. The fetal heart generates most of its ATP using acetyl CoA derived from glucose and lactate oxidation, with only a minor contribution from fatty acids. After birth, this switches so the increased demand for ATP in the mature heart is met primarily via oxidation of acetyl CoA generated from mitochondrial fatty acid oxidation (Lopaschuk & Jaswal, 2010). Carnitine palmitoyltransferase I (CPT-1) transports long-chain fatty acids such as palmitate into mitochondria where they are oxidized by a series of enzymes including long-chain and medium-chain acyl dehydrogenases (LCAD and MCAD, respectively) to acetyl CoA which can then enter the TCA cycle. The increase in fatty acid oxidation is associated with greater mitochondrial functional capacity. PGC-1α, a master transcriptional regulator of mitochondrial capacity, is expressed in the late gestation fetal heart and expression rises markedly after birth (Lehman *et al.* 2000). Mice with global knockout of PGC-1α show 50% mortality before weaning (Lin *et al.* 2004), suggesting that it is important in the perinatal period. PGC-1α is a glucocorticoid target gene and, *in vivo* in fetal heart, is induced 6 h after glucocorticoid treatment in mice (Rog-Zielinska *et al.* 2015). PGC-1α is also induced *in vitro* in primary mouse fetal cardiomyocytes (Rog-Zielinska *et al.* 2015). Here, the GR-mediated increase in PGC-1α expression is crucial for the glucocorticoid-induced maturation of myofibril structure and increased mitochondrial O_2 consumption (Rog-Zielinska *et al.* 2015). Knock-down of PGC-1α abolished both. RNAseq analysis performed on primary mouse fetal cardiomyocytes harvested 2 h after glucocorticoid addition in the presence of cycloheximide (to block secondary effects) identified a number of differentially expressed genes, likely to be primary targets of GR (Rog-Zielinska *et al.* 2015). As well as *Ppargc1a* (encoding PGC-1α), master regulators of mitochondrial fatty acid oxidation (*Klf15, Lipin1, Cebpb, Ppara*) were induced. This suggests that glucocorticoid action may promote the perinatal switch in cardiomyocytes from carbohydrate to fatty acid oxidation as the preferred substrate for ATP generation.

Cellular differentiation is often associated with metabolic remodelling and the autophagic turnover of mitochondria by mitophagy (Rodger *et al.* 2018). Mitophagic removal of small fetal mitochondria in perinatal cardiomyocytes is reportedly a prerequisite for the formation of morphologically distinct adult mitochondria and maturation into cardiomyocytes optimized for fatty acid metabolism (Gong *et al.* 2015). The triggers for mitophagy in perinatal cardiomyocytes are currently unknown. *mito*-QC transgenic mice have a pH-sensitive fluorescent mitochondrial signal that monitors mitophagy *in vivo* (McWilliams *et al.* 2016). These mice have revealed that mitophagy is occurring at E17.5 in the mouse fetal heart (McWilliams *et al.* 2016), a time coincident with peak GR activation in heart (Rog-Zielinska *et al.* 2013). Furthermore, *Bnip3*, implicated in mitophagy, is a direct GR target gene in primary fetal cardiomyocytes (Rog-Zielinska *et al.* 2015), raising the possibility that glucocorticoids may be a trigger for mitophagy in perinatal cardiomyocytes.

Here, we hypothesized that glucocorticoids increase fetal cardiomyocyte capacity for fatty acid oxidation. We also asked if any glucocorticoid-mediated increase in mitochondrial fatty acid oxidation capacity involves mitochondrial remodelling by mitophagy.

Materials and methods

Ethical approval

All experiments on mice were carried out in strict accordance with accepted standards of humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986. Experiments involving *mito*-QC mice were carried out under the authority of UK Home Office Project Licence PA3BFDB52 following approval by the University of Dundee ethical review committee with further study plan approval by the Named Veterinary Surgeon and Compliance Officer. Experiments involving all other mice were carried out under the authority of UK Home Office Project Licence P359AF310 following approval by the University of Edinburgh Animal Welfare and Ethical Review Body with further approval of the experimental study plans by the Named Veterinary Surgeon. Sheep experimentation and protocols were approved by the animal ethics committee of The University of Western Australia (RA/3/100/1452) and maintained the standards of the *Australian code for the care and use of animals for scientific purposes*, 8th edition 2013.

Animals

Mice were maintained under controlled lighting and temperature with *ad libitum* access to food and water. C57BL/6J/Ola/Hsd (C57Bl/6J) mice were purchased from Harlan, then bred in-house. $GR^{+/-}$ mice, heterozygous for a null mutation in the *Nr3c1* gene encoding GR ($Nr3c1^{gtESK92MRCHGU}$ mice), have been previously described (Michailidou *et al.* 2008; *Rog*-Zielinska *et al.* 2013). $GR^{+/-}$ mice, congenic on the C57Bl/6J background (>12 generations) were intercrossed to

give $GR^{+/+}$, $GR^{+/-}$ and $GR^{-/-}$ fetal littermates. The morning of the day the vaginal plug was found was designated E0.5. Fetuses were collected at E17.5 and killed by decapitation, hearts were dissected and rapidly frozen on dry ice. Genotyping of fetal tissue by PCR used *LacZ* primers for the $Nr3c1^{gtESK92MRCHGU}$ (5'-GAGTTGCGTGACTACCTACGG-3' and 5'-GTACCACAGCGGATGGTTCGG-3') and wild-type *GR* alleles as described (Michailidou *et al*. 2008). *mito*-QC mice, also on a C57Bl/6J background, have been described (McWilliams *et al*. 2016). *mito*-QC heterozygous fetuses were used for fetal cardiomyocyte cultures. Fetuses were collected and cardiomyocytes isolated as described below.

For dexamethasone treatment, pregnant C57Bl/6J females (time-mated with C57Bl/6J males) were semi-randomized to experimental group (n = 5/group/time point; alternating between groups as lifted from the cage) and injected (∼0.1 ml, I.P.) with dexamethasone (0.5 mg/kg; Sigma-Aldrich, Poole, UK) or the vehicle used to dissolve dexamethasone (5% ethanol) at either E13.5 or E16.5 and killed 24 h later (E14.5 and E17.5, respectively) by cervical dislocation. Fetuses were removed to ice-cold PBS and killed by decapitation. Hearts were excised and frozen on dry ice. For analysis of fetal heart RNA, one or two animals per litter were analysed (where two animals from a litter were analysed, the mean of the two was used), so n = 5. Neonatal C57Bl/6J mice were injected (I.P.) on postnatal day 1 (P1; day of birth being P0) with dexamethasone (0.5 mg/kg) or vehicle (5% ethanol) and killed by decapitation (at P2) 24 h later (n = 7 vehicle-treated neonates from seven different litters, n = 8 dexamethasone-treated neonates from eight different litters). Hearts were removed and frozen as above. Pregnant $GR^{+/-}$ dams were killed at E17.5 by cervical dislocation. For RNA analysis, fetal hearts were removed and frozen as above (n = 5–6 $GR^{+/+}$ or $GR^{-/-}$ fetuses, from the litters of six dams) for later RNA extraction. A different cohort of six pregnant $GR^{+/-}$ dams were used for mitochondrial DNA quantification with n = 6 $GR^{+/+}$ and $GR^{-/-}$ fetuses, each from a different litter; fetal hearts were removed and frozen for later DNA extraction. All tissues were identified by animal ID (blinding to genotype/treatment group) and stored at -80°C prior to analysis. Merino ewes were obtained from Icon Agriculture, Darkan, Western Australia, Australia and had free access to food and water throughout. Prior to delivery, animals were maintained in an outdoor field environment with free access to water and grazing. Supplemental feed (chaff, oaten hay, sheep pellets) was provided twice daily. Date-mated ewes carrying singleton pregnancies were randomized to receive two injections (intramuscular) on days 120 and 121 of gestation, spaced by 24 h of either saline (control; celestone is formulated in buffered saline) or betamethasone acetate with betamethasone phosphate (celestone chronodose, Merck & Co., Inc, Kenilworth, NJ) 0.25 mg/kg per injection; n = 9/group (celestone), n = 11/group (vehicle). A third group (n = 10) received a single injection of betamethasone acetate (0.125 mg/kg). Betamethasone acetate was a gift from Merck & Co. as a preparation of betamethasone acetate equivalent to that in celestone. Merck & Co. did not participate in the design, execution or analysis of the study. The 0.25 mg/kg celestone dose approximates the clinical dose of 12 mg of betamethasone for a 50 kg woman and was the same dose used for our previous studies (Kemp *et al*. 2018; Schmidt *et al*. 2019a). To reduce the risk of steroid-induced preterm labour from antenatal corticosteroids, all pregnant ewes, both steroid-treated and control animals, received one intramuscular dose of 150 mg medroxyprogesterone acetate (Depo-Provera, Pfizer, New York, NY) on day 115 of gestation, 5 days before corticosteroid treatment. No other doses of medroxyprogesterone acetate were administered, nor were other tocolytics administered. All animals were delivered at 122 ± 1 days (term being ∼147 days).

Two days after their initial steroid or saline treatment, on day 122 of gestation, ewes received an intravenous injection of ketamine (10 mg/kg) and midazolam (0.5 mg/kg). A spinal injection of 3 ml lignocaine (20 mg/ml) was then administered and surgical delivery commenced. Ewes were killed under anaesthesia at the time of delivery. The lamb received an intramuscular injection of ketamine (10 mg/kg) before placing a 4.5 mm endotracheal tube by tracheostomy. Lambs were weighed, dried, and placed in an infant warmer (Fisher & Paykel Healthcare, New Zealand). Intermittent positive pressure ventilation was performed using Acutronic Fabian infant ventilators (Acutronic Medical System, Hirzel, Switzerland) and maintained for 30 min using the following settings: initial peak inspiratory pressure (PIP) of 40 cmH_2O, positive end expiratory pressure of 5 cmH_2O, respiratory rate of 50 breaths per minute, inspiratory time of 0.5 s. Gas mix was 100% heated and humidified oxygen. PIP was titrated to achieve a tidal volume of 7 ml/kg. Lambs were ventilated to assess preterm lung function in response to antenatal steroid exposure as part of a separate study. The ventilation procedure was brief (30 min) and applied to both control (saline) and steroid-exposed animals. Lambs were killed with an IV overdose of pentobarbitone sodium at 160 mg/kg. Necropsy was performed immediately following a 30 min ventilation procedure which commenced at delivery. At necropsy (within 40 min of delivery), left and right ventricles of the heart were dissected and samples snap frozen.

Fetal cardiomyocyte cultures. Primary fetal cardiomyocyte cultures were prepared as described (Rog-Zielinska *et al*. 2015). Briefly, hearts were rapidly

dissected from 15–30 E14.5–E15.5 C57Bl/6J or *mito*-QC fetuses. The number of fetuses differed between each cell culture study, but pools always contained between 15 and 30 fetal hearts. E14.5 and E15.5 fetal hearts (after initiation of fetal adrenal steroidogenesis) were combined to minimize animal wastage; there is no discernible difference between wild-type and GR-deficient mice at either time point (Rog-Zielinska *et al*. 2013). Pooled hearts were placed in warm Tyrode's salt solution containing 0.1% sodium bicarbonate then rinsed in complete medium (DMEM supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 10% fetal bovine serum, 0.1% non-essential amino acids, Sigma-Aldrich, Poole, UK) before digestion at 37°C with gentle agitation for 10 min in 5 ml enzyme buffer (PBS supplemented with 0.8% NaCl, 0.2% D-(+)-glucose, 0.02% KCl, 0.000575% $NaH_2PO_4.H_2O$, 0.1% $NaHCO_3$, pH 7.4) containing 0.03% type II collagenase (Worthington Biochemical Corp, Lakewood, New Jersey, USA), 0.125% porcine pancreatin (Sigma-Aldrich, Poole, UK). After 10 min, isolated cells and enzyme buffer were removed and the enzymatic reaction quenched by adding the same volume of complete medium. Fresh enzyme buffer was then added to the hearts. Approximately eight digestions were performed on the hearts until their structure was lost. Isolated cells were centrifuged at 1000 g for 10 min at room temperature, the supernatant removed and pellets pooled. Pooled cells were centrifuged again and resuspended in 15 ml isolation buffer (Ham's F12 supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.002% ascorbic acid, 1% fetal bovine serum, 0.1176% $NaHCO_3$). To reduce the number of fibroblasts, cells were incubated in a tissue culture plate (ThermoFisher, UK) at 37°C, 5% CO_2 for 3 h during which fibroblasts adhered to the plastic. Non-adherent cells were aspirated, centrifuged, resuspended in 1 ml of complete medium and seeded at a density of 0.25 $\times$ 10^6 cells/ml for extracellular flux (ECF; seahorse) assays, RNA analysis or mitochondrial morphology. This protocol yields $\geq$98% cardiomyocytes (troponin T^+ cells) (Rog-Zielinska *et al*. 2015). Spontaneous beating of cardiomyocytes was observed within 12 h. For ECF assays, cardiomyocytes were seeded onto 24-well gelatin (Sigma, UK) coated V7 seahorse plates (Agilent Technologies LDA UK Ltd, Stockport, Cheshire, UK) in complete medium then treated with dexamethasone (1 μM) or vehicle (0.01% ethanol) for 24 h prior to ECF assay (below). We have previously shown that this dose of dexamethasone elicits maximal glucocorticoid responses in fetal cardiomyocytes (Rog-Zielinska *et al*. 2015). For RNA analysis, primary fetal cardiomyocytes were seeded in 12-well gelatin-coated tissue culture plates for 48 h. Cells were treated with dexamethasone (1 μM) or vehicle (0.01% ethanol) and lysed 6 or 24 h later by adding 0.5 ml TRIzol (Invitrogen, ThermoFisher, UK) following removal of medium. For measurement of mitochondrial morphology and mitophagy, cardiomyocytes were cultured on gelatin-coated glass chambered slides (Ibidi μ-slide 4-well glass bottom, Thistle Scientific LTD, Glasgow, UK) prior to staining.

Extracellular flux assays. ECF assays were carried out using a Seahorse XF^e24 Bioanalyzer (Agilent Technologies LDA UK Ltd, Stockport, Cheshire, UK). All seahorse reagents were purchased from Agilent Technologies LDA UK Ltd. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured. The basal OCR reflects mitochondrial oxidative phosphorylation, non-mitochondrial cellular metabolism (e.g. dioxygenases), ROS production and proton leak (basal mitochondrial respiration not coupled to ATP production). The ECAR reflects lactate released as a product of glycolysis and also acidification by mitochondrial production of CO_2. Inhibitors were used to assess the contributions of glycolysis and mitochondrial complexes to ECAR and OCR, respectively. 2-deoxyglucose (2DG) is phosphorylated by hexokinase, the first and rate-limiting enzyme in glycolysis, but cannot be further metabolized, so inhibits glycolysis. Oligomycin blocks the proton channel of ATP synthase (complex V), thus preventing oxidative phosphorylation of ADP to ATP, providing a measure of mitochondrial ATP generation. Rotenone and antimycin A inhibit complex I and complex III of the mitochondrial electron transport chain, respectively, completely blocking mitochondrial electron transport and thus mitochondrial respiration. Any residual oxygen consumption following addition of antimycin A and rotenone (AR) is independent of mitochondrial respiration. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) allows protons through the mitochondrial membrane, uncoupling mitochondrial electron transport from proton transfer and ATP generation. FCCP thus permits electron transport and oxygen consumption by complex IV to proceed maximally, providing a measure of mitochondrial reserve capacity. For each experiment, treatment groups were randomized across the plate. Each well was assigned a number (based on the number of treatment groups) so that numbers were spread across the plate. Treatments were then randomized to numbers. On the day of the assay, complete culture medium was gently aspirated from the cells and exchanged for seahorse assay medium (with supplements defined below). The cells were gently rinsed three times with seahorse assay medium, leaving a final volume of 525 μl for the assay. During all assays, three measurements at 2.5 min intervals, were recorded at baseline and after each drug addition. Each figure shows data from a different experiment or experiments.

Normalization of extracellular flux assays using sulforhodamine B assay. ECF assays were normalized to protein measured using sulforhodamine B (SRB) dye-based protein assay (Skehan *et al.* 1990). Initial experiments confirmed the linearity of the SRB assay for use in quantifying primary cardiomyocyte protein levels ($R^2 = 0.93$; Fig. 1*A*, $n = 3$). Following ECF assays, cells were fixed by addition of 50 μl cold 50% trichloroacetic acid (Sigma-Aldrich, Poole, UK) per well and stored for up to 1 week at 4°C. Cells were then washed 10 times with tap water and air-dried. Fifty microlitres of SRB solution (0.4% w/v sulforhodamine B dye (Sigma-Aldrich, Poole, UK) in 1% acetic acid (Sigma-Aldrich, Poole, UK)) was added to dried cells and incubated for 30 min at room temperature. Cells were washed four times with 1% acetic acid and air-dried. Cell-bound dye was redissolved in 200 μl 10 mM Tris pH 10.5. Absorbance was measured at a wavelength of 540 nm. There was no difference in protein content, measured by SRB assay, between dexamethasone and vehicle-treated cells (2.82 ± 0.0373 vehicle *vs.* 2.83 ± 0.0374 dexamethasone, $n = 10$ wells/treatment from one experiment, $P = 0.803$ by *t* test).

Glycolysis assays. Assays to estimate the rate of glycolysis in fetal cardiomyocytes were performed in two different ways. In the first, a glycolysis stress test was performed according to the manufacturer's protocol. Briefly, the cells were incubated in pre-warmed glucose-free seahorse-XF base medium for 1 h in a (non-CO_2) 37°C incubator prior to the assay. After three basal measurements, glucose (Sigma-Aldrich, Poole, UK, 10 mM) was added to enable glycolysis, followed by oligomycin (Sigma-Aldrich, Poole, UK, 1.5 μM) to inhibit respiratory ATP production and finally 2DG (Sigma-Aldrich, Poole, UK, 100 mM) to inhibit glycolysis. In the second, complete culture medium was exchanged for pre-warmed seahorse assay medium supplemented with 10 mM glucose (Sigma-Aldrich, Poole, UK), 1 mM sodium pyruvate (Sigma-Aldrich, Poole, UK). After basal OCR/ECAR measurements, 2DG (Sigma-Aldrich, Poole, UK, 100 mM) was added to inhibit glycolysis, followed by AR (Sigma-Aldrich, Poole, UK, 2 μM) to inhibit the electron transport chain.

Fatty acid oxidation assay. To test the ability of primary fetal cardiomyocytes to utilize long-chain fatty acids, cells were pre-treated with etomoxir (Sigma-Aldrich, Poole, UK) to inhibit the CPT-1 mitochondrial fatty acid uptake transporter, prior to performing a standard seahorse mitochondrial stress test (Fig. 1*B*). Initial experiments with high concentrations of etomoxir (40–160 μM) reduced OCR in the presence of bovine serum albumin (BSA)-palmitate (Fig. 1*C*). However, within this range (120 μM), etomoxir also showed likely off-target inhibition of mitochondrial respiration (Fig. 1*B*). This is consistent with an emerging literature showing that etomoxir can inhibit complex I at commonly used high doses (Divakaruni *et al.* 2016; Yao *et al.* 2018). Accordingly, we used 6 μM etomoxir for all further experiments, a dose which inhibits fatty acid oxidation and avoids the CPT-1-independent effects associated with higher doses (Spurway *et al.* 1997). Briefly, culture medium was exchanged for seahorse assay medium supplemented with 5 mM glucose (Sigma-Aldrich, Poole, UK), 0.5 mM carnitine (Sigma-Aldrich, Poole, UK). Cells were pre-treated with etomoxir (6 μM in medium) or vehicle (medium) 15 min prior to the addition of BSA-palmitate (100 μM; Agilent Technologies LDA UK Ltd). The seahorse mitochondrial stress test assay was started 15 min after the addition of BSA-palmitate. Briefly, the test progressed as follows: basal respiration measurements, addition of oligomycin (1.5 μM), addition of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 1 μM), addition of AR (2 μM). Non-mitochondrial respiration was calculated as the minimum OCR remaining following AR treatment. The mean of the three baseline measurements was used for the basal OCR. Basal respiration was calculated by subtracting non-mitochondrial respiration from basal OCR. ATP production was calculated as the maximum change in OCR following the addition of oligomycin and maximum respiration was calculated as the maximum OCR measurement induced by FCCP corrected for non-mitochondrial respiration. Leak respiration was calculated as the average oligomycin-insensitive OCR corrected for non-mitochondrial respiration.

Measurements of mitochondrial morphology. On the day of staining of primary fetal cardiomyocytes, complete culture medium was exchanged for serum-free medium. MitoTracker Deep Red (40 nM; ThermoFisher, UK) was added and incubated for 30 min in cell culture conditions. Serum-free medium was then replaced. To image cardiomyocytes as a z stack, beating was stopped by adding 100 mM nefidipine (Sigma-Aldrich, Poole, UK) immediately prior to imaging using an Andor Spinning Disk confocal microscope. The Andor Spinning Disk system is based on an inverted Olympus IX83 microscope stand and a Yokogawa CSU-X1 spinning disk module. It is equipped with an Oko Labs environmental control chamber to maintain stable conditions for live cell imaging; 37°C, 5% CO_2 were used throughout. To acquire GFP and mCherry images, 488 nm (BP525/25) and 561 nm (LP568), respectively, laser lines (and emission filters) operated via an AOTF were used. A plan super apochromat 100 × 1.4 NA oil immersion objective was used throughout, with z steps of 1 μm taken. Images were acquired onto an Andor iXon Ultra EMCCD camera (512 × 512) using an EM gain of 200 with

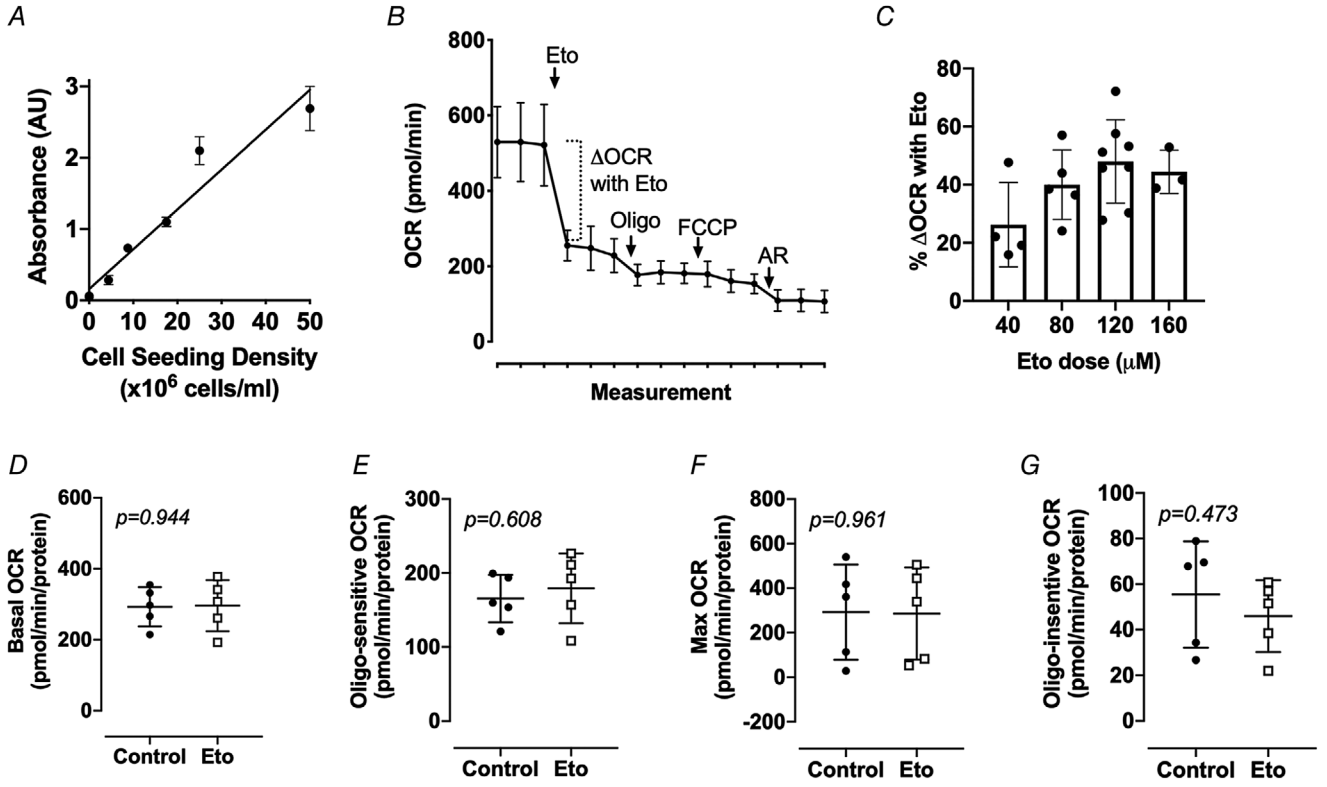

Figure 1. Optimization experiments for extracellular flux assays in primary fetal cardiomyocytes
All experiments were performed using primary fetal cardiomyocytes prepared by digesting 15–30 pooled C57BL6/J fetal hearts at E14.5–15.5 and cultured for 48 h in complete DMEM culture medium. *A*, the sulpho-rhodamine assays, used to estimate protein levels for normalization of extracellular flux assays, was linear over a range of primary fetal cardiomyocyte seeding densities, n = 3 wells of pooled cells, line fitted by linear regression with R-square value of 0.93. *B*, cell culture complete medium was exchanged for seahorse assay medium (supplemented with 1 mM GlutaMAX, 10 mM glucose, 1 mM pyruvate and 0.5 mM carnitine). Cardiomyocytes were treated with BSA-palmitate (100 μM) 30 min prior to extracellular flux analysis. After three basal measurements, Etomoxir (Eto) was added (120 μM), followed by oligomycin (1.5 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μM) and antimycin and rotenone (AR, 2 mM). After Eto-treatment, these cells failed to uncouple in response to FCCP and exhibited a very small response to AR consistent with the emerging literature that Eto can inhibit complex I and III at high concentrations. *C*, cardiomyocytes were treated with BSA-palmitate (100 μM) in seahorse assay medium 30 min prior to extracellular flux analysis. The change in oxygen consumption rate (OCR) with increasing doses of Eto was measured (indicated in panel (*B*) by a dotted line). Dose response experiments with increasing doses of Eto indicated that doses of 40–120 μM gave similar % changes in OCR, n = 3–5 wells of pooled cells. *D–G*, finally, the effect of Eto in the absence of the fatty acid palmitate was investigated. Cell culture complete medium was exchanged for seahorse assay medium (supplemented with 1 mM GlutaMAX, 5 mM glucose and 0.5 mM carnitine). Cells were treated with Eto (6 μM) or vehicle (Control, medium) 15 min prior to the addition of BSA alone (17 μM). After 15 min incubation, cardiomyocyte metabolism was analysed by extracellular flux assays. After three basal measurements, oligomycin was added (1.5 μM) followed by carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μM) and AR (2 mM). OCR (*A*) was measured in triplicate for 2.5 min following each drug addition. Non-mitochondrial respiration was estimated as the average OCR remaining after treatment with AR. *D*, basal respiration was estimated as the mean of the three basal OCR measurements corrected for non-mitochondrial respiration. *E*, ATP production was estimated as the maximum change in OCR following the addition of oligomycin. *F*, maximum respiration was estimated by the maximum OCR measurement induced by FCCP corrected for non-mitochondrial respiration. *G*, leak respiration was estimated as the average oligomycin-insensitive OCR corrected for non-mitochondrial respiration. Data are means ± SD, data analysed by Student's t tests, n = 5 individual wells of primary fetal cardiomyocytes pooled from 15–30 fetal hearts analysed at the same time across one experiment.

50 ms exposure. Mitochondrial volume was quantified using open-source MitoGraph software (Viana *et al.* 2015; Harwig *et al.* 2018). This uses 3D reconstructions of labelled organelles to measure the morphology of individual mitochondria as well as characteristics of the mitochondrial network and provides the following outputs: mitochondrial volume, total length and average width. Post-image processing and MitoGraph analysis was performed as per the online protocols (available: http://rafelski.com/susanne/MitoGraph). Between 17 and 40 individual cardiomyocytes from one experiment were quantified in the data shown here (a replicate experiment with the same findings is not reported).

Mitophagy assay. Primary fetal cardiomyocyte cultures prepared from E14.5–15.5 *mito*-QC fetuses were seeded at a density of 0.25×10^6 cells/ml on gelatin-coated glass chambered slides (Ibidi μ-slide 4-well glass bottom, Thistle Scientific LTD, Glasgow, UK). After 48 h, cells were treated with 1 μM dexamethasone, 1 mM deferiprone (DFP, an iron-chelator, used as a positive control) or vehicle (0.01% ethanol). After 24 h dexamethasone/DFP/vehicle-treated cells were imaged live and z stacks were generated 3, 8 and 45 h after treatment, using the Andor Spinning Disk confocal live cell imaging system as above. Post-acquisition image analysis was performed blind to the treatment. For each cell, a z stack was acquired and red puncta, indicative of mitophagy, were counted.

RNA extraction. Primary fetal cardiomyocytes seeded in 12-well plates were lysed in 500 μl TRIzol (Invitrogen, ThermoFisher, UK) and aspirated into a 1.5 ml tube. Chloroform (Sigma-Aldrich, Poole, UK; 100 μl) was added and samples vigorously shaken for 30 s. Samples were incubated for 2–3 min at room temperature then centrifuged 10,000 g for 15 min at 4°C. The aqueous phase was transferred to a new tube containing 250 μl isopropanol (Sigma-Aldrich, Poole, UK) and centrifuged as above. The supernatant was discarded and the pellet washed with 500 μl 70% ethanol twice. Samples were centrifuged at 10,000 g for 10 min at 4°C, the supernatant removed and pellet air-dried at room temperature for 5–10 min before resuspension in 30 μl RNAse-free water.

Mouse fetal hearts were individually homogenized using a stainless steel bead in 500 μl RLT buffer (RNeasy, Qiagen, Manchester, UK) and 1% β-mercaptoethanol with a TissueLyser II (Qiagen, Manchester, UK) at maximum speed for 2 min. Ten microliters of proteinase K (20 mg/ml; Qiagen, Manchester, UK) and RNAse-free water were added (final volume of 900μl). Samples were incubated at 56°C for 10 min, centrifuged at 10,000 g for 5 min at room temperature and the supernatant transferred to a fresh tube containing 400 μl 96% ethanol. The mixture was transferred to RNeasy spin tubes (Qiagen mini prep), processed according to the manufacturer's instructions and eluted in 20 μl RNAse-free water. This was incubated on the column for at least 5 min at room temperature before collection. The first eluate was reapplied to the column and incubated for a further minute before the final elution.

For sheep hearts, RNA extraction was similar except that samples were minced prior to lysis for two sessions each of 3 min.

RNA quantity and integrity were determined using a Nanodrop (ThermoFisher, UK) spectrophotometer and gel electrophoresis, respectively.

Reverse transcription and quantitative real-time PCR. Samples of 500 ng mouse or 300 ng sheep RNA were reverse transcribed using QuantiTect Reverse transcription kit (Qiagen, Manchester, UK) and gDNA wipe-out (to remove genomic DNA), according to the manufacturer's protocol. Included in each sample batch were 'no template' and 'no reverse transcriptase' controls. Resultant cDNA samples were stored at -20°C. Primers and probes used for qPCR are detailed in Table 1. Assays for mouse qPCR were designed using the Roche Universal Probe Library and primers were purchased from Invitrogen (ThermoFisher, UK). A standard curve prepared from pooled cDNA samples was processed with samples on a Lightcycler 480 system (Roche Diagnostics, Burgess Hill, UK). For sheep, Taqman assays (ThermoFisher, UK) were used and performed using a 7900HT Fast Real Time PCR system (ThermoFisher, UK). Internal controls were *Tbp* for mouse, and *PGK1* and *SDHA* for sheep heart; these did not differ across treatments. All assays were performed in triplicate. Quantification was achieved using the PCR machine software, which calculated $2\Delta\Delta$CT by comparison of unknown sample values to an external standard curve. Standard curves passed quality control if they had efficiency within the range of 1.7–2.1 and error of <0.5). To normalize the spread, data were $\log_{10}$ transformed. For mouse RNA data, each data point represents a single litter; either one fetal heart or the mean of the measurements from two fetal hearts.

Mitochondrial DNA quantification. DNA was extracted from frozen samples ($n = 6$ fetal hearts/genotype) using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol. To quantify mitochondrial DNA relative to nuclear DNA, levels of mitochondrial-encoded genes (*Co1, Co2, Nd2*) were measured relative to an intronless nuclear-encoded gene (*Cebpa*) by qPCR, performed in triplicate (primer sequences in Table 1). At E17.5, cardiomyocytes in mouse fetal hearts are mono-nucleated (Rog-Zielinska *et al.*

Table 1. Sequences of primers and probes used in real-time PCR

Assay	Comment	Accession number	Forward primer 5′-3′	Reverse primer 5′-3′	UPL probe
Rn18s	18S RNA	NR_003278.1	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG	77
Tbp	TATA box binding protein	NM_013684	GGGAGAATCATGGACCAGAA	GATGGGAATTCCAGGAGTCA	97
Nr3c1	Glucocorticoid receptor (mouse)	NM_008173.4	TGACGTGTGGAAGCTGTAAAGT	CATTTCTTCCAGCACAAAGGT	56
Fkbp5	FK509 binding protein	NM_010220.4	AAACGAAGGAGCAACGGTAA	TCAAATGTCCTTCCACCACA	97
Ppargc1a	PGC-1α (mouse)	NM_008904.2	GAAAGGGCCAAACAGAGAGA	GTAAATCACACGGCGCTCTT	29
Acadm (Mcad)	Medium-chain specific acyl-CoA dehydrogenase	NM_007382.5	GGAAAGCTGCTAGTGGAGCA	TGCAATCGAGGCATAGTAAGTG	64
Acadl (Lcad)	Long-chain specific acyl-CoA dehydrogenase	NM_007381.4	AGTGTATCGGTGCCATAGCC	TGGCGTTCGTTCTTACTCCT	11
Lpin1 (Lipin1)	Lipin1	NM_172950.3	GACTGGGAAAGGCCACAATA	CGTGCTCTTCATCACTGGAG	49
Cd36	CD36	NM_001159555	TTGAAAAGTCTCGGACATTGAG	TCAGATCCGAACACAGCGTA	6
Cpt1a	Carnitine pamitoyltransferase-1α	NM_013495.2	CTCAGTGGGAGCGACTCTTC	TGTCCTTGACGTGTTGGATG	94
Cpt1b	Carnitine pamitoyltransferase-1β	NM_009948.2	GTACCGCCTAGCCATGACA	GGCTCCAGGGTTCAGAAAGT	29
Ucp2	Uncoupling protein 2	NM_011671.5	ACAGCCTTCTGCACTCCTG	GGCTGGGAGACGAAACACT	2
Mt-CO1	Mitochondrial-encoded cytochrome oxidase-1 (mitochondrial genomic DNA)	N/A	CAGACCGCAACCTAAACACA	TTCTGGGTGCCCAAAGAAT	25
Mt-CO3	Mitochondrial-encoded cytochrome oxidase-2 (mitochondrial genomic DNA)	N/A	TAGCCTCGTACCAACACATGA	AGTGGTGAAATTCCTGTTGGA	66
Mt-ND2	Mitochondrial-encoded NADH dehydrogenase-2 (mitochondrial genomic DNA)	N/A	CAAATTTACCCGCTACTCAACTC	GCTATAATTTTTCGTATTTGTGTTTGG	101
Cebpa	Nuclear-encoded C/EBPα (intronless gene)	TaqMan ABI Mm_00514283_s1			
NR3C1	Glucocorticoid receptor (sheep)	TaqMan ABI Oa_04657790_m1			
PPARGC1A	PGC-1α (sheep)	TaqMan ABI Oa_01208835_m1			
PGK1	Phosphoglycerate kinase-1 (sheep)	TaqMan ABI Oa_04657427_gH			
SDHA	Succinate dehydrogenase subunit A	TaqMan ABI Oa_04307499_m1			

Forward and reverse primers and Roche Universal Probe Library (Invitrogen, ThermoFisher, UK) probe number used in real-time PCR assays (mouse, unless specified otherwise) to measure specific cDNA/mitochondrial DNA levels.

2013). Moreover, dexamethasone treatment of mouse fetal cardiomyocytes *in vitro* has no effect on nucleation (Rog-Zielinska *et al.* 2015).

Statistics. Graphpad Prism 8 software was used for statistical analyses. All data are presented as means ± standard deviation (SD). The number of biological replicates is provided in the figure legends together with the statistical tests used for analysis. All data were subject to Shapiro–Wilk's normality testing prior to analysis. Outliers were identified using Grubbs' test. Two outliers in total were detected and excluded from the data analysis (as detailed in the legends to Figs 8 and 9). Parametric analyses, Student's *t* tests and two-way ANOVA with *post hoc* Sidak's tests were used as stated in the figure legends.

Results

Primary fetal cardiomyocytes rely mainly on mitochondrial oxidation, with little reliance on glycolysis

The OCR and ECAR were measured in a glycolysis stress test (without fatty acid). The basal OCR prior to, and following, addition of 10 mM glucose was increased by 24 h dexamethasone treatment of primary fetal cardiomyocytes (Fig. 2*A*, 2*C*; $P = 0.0247$, $n = 9 = 10$). OCR was markedly decreased following addition of oligomycin, an inhibitor of ATP synthase (Fig. 2*A*), suggesting high dependence of fetal cardiomyocytes on mitochondrial respiration for ATP production. There was a modest increase in oligomycin-sensitive OCR (basal respiration) in fetal cardiomyocytes treated with dexamethasone, consistent with our previous findings (Rog-Zielinska *et al.* 2015) though this did not achieve significance here (Fig. 2*D*, $P = 0.0729$, $n = 9 = 10$). The ECAR is a key measure of glycolysis, an important energy source in early fetal cardiomyocytes (Porter *et al.* 2011). However, our murine primary fetal cardiomyocytes exhibited little dependence on glycolysis. The addition of 2DG (an inhibitor of glycolysis) had a minimal impact on ECAR when added either during the glycolysis stress test (Fig. 2*B*) or when added to glucose-replete medium without any other inhibitors (Fig. 2*F*, 2*G*; $P = 0.765$, $n = 5$) and was unaffected by dexamethasone treatment (Fig. 2*E*; $P = 0.103$, $n = 9 = 10$). As expected, addition of the glycolysis inhibitor 2DG did not alter the OCR (Fig. 2*A*). These data suggest primary fetal cardiomyocytes perform very little glycolysis, relying mainly on mitochondrial oxidation for ATP production.

Dexamethasone increases palmitate oxidation via GR activation

To investigate whether dexamethasone can increase capacity for long-chain fatty acid oxidation in fetal cardiomyocytes, OCR was measured in the presence of palmitate in a mitochondrial stress test. Long-chain fatty acids are linked to carnitine and transported into the mitochondrial matrix by carnitine palmitoyltransferase-1 (CPT-1), which is inhibited by etomoxir. Following treatment of primary fetal cardiomyocytes with dexamethasone for 24 h, in the presence of palmitate there was an increase in basal OCR (Fig. 3*A*, 3*B*; $P = 0.00638$, $n = 4–5$). Furthermore, in the presence of palmitate, dexamethasone-treated cardiomyocytes showed a larger change in OCR following oligomycin treatment (oligomycin-sensitive OCR; Fig. 3*C*; $P = 0.0228$, $n = 4–5$), indicative of increased mitochondrial ATP production. Addition of etomoxir attenuated the effect of dexamethasone on basal OCR and mitochondrial ATP production (oligomycin-sensitive OCR), so that any residual effect did not achieve significance (Fig. 3*A*–3*C*; $P = 0.108$ and $P = 0.240$, respectively, $n = 4–5$). This suggests that dexamethasone increased palmitate oxidation in fetal cardiomyocytes. Etomoxir itself had no effect on respiration ($P = 0.944$), ATP production ($P = 0.608$) or maximum respiration capacity ($P = 0.961$) by fetal cardiomyocytes in the absence of palmitate (Fig. 1*D*–1*F*, $n = 5$) nor did it affect leak respiration (Fig. 1*G*; $P = 0.473$, $n = 5$). The dexamethasone-induced increase in fatty acid oxidation was dependent on GR, as pre-treatment of the cardiomyocytes with the GR antagonist, RU486 blocked the increase in basal respiration and ATP production (Fig. 4*A*–4*C*; P[interaction] $= 0.0357$ and $P = 0.0493$, respectively, $n = 12$).

Dexamethasone upregulates genes involved in long-chain fatty acid oxidation in fetal cardiomyocytes

The increase in ability to utilize palmitate as a fuel for mitochondrial respiration suggests that glucocorticoids increase mitochondrial capacity for long-chain fatty acid oxidation. Consistent with the rapid induction of PGC-1α and other master regulators of lipid metabolism in dexamethasone-treated fetal cardiomyocytes (Rog-Zielinska *et al.* 2015) there was a marked induction of mRNAs encoding enzymes and transporters required for mitochondrial fatty acid oxidation in primary fetal cardiomyocytes after treatment with dexamethasone (Fig. 5). As well as the master transcriptional regulators, *Ppargc1a* and *Lipin1*, 24 h treatment with dexamethasone induced expression of *Lcad* ($P < 0.0001$; $n = 4$) and *Mcad* ($P = 0.000308$; $n = 4$) (encoding, respectively, long-chain acyl dehydrogenase and medium-chain acyl dehydrogenase), *Cd36* ($P < 0.0001$; $n = 4$), encoding cluster of differentiation-36, also known as fatty acid translocase (a cellular importer of fatty acids), and *Cpt1a*

and *Cpt1b* ($P < 0.0001$; $n = 4$), encoding the alpha and beta subunits, respectively, of CPT-1 (Fig. 5*B*). Dexamethasone also increased expression of *Ucp2* ($P = 0.00132$; $n = 4$), encoding uncoupling protein 2, an inner mitochondrial membrane protein that promotes mitochondrial fatty acid oxidation at the expense of mitochondrial catabolism of pyruvate (Pecqueur *et al.* 2008). At just 6 h after addition of dexamethasone, although *Fkbp5*, a well-known glucocorticoid target was strongly upregulated ($P < 0.0001$; $n = 4$), levels of

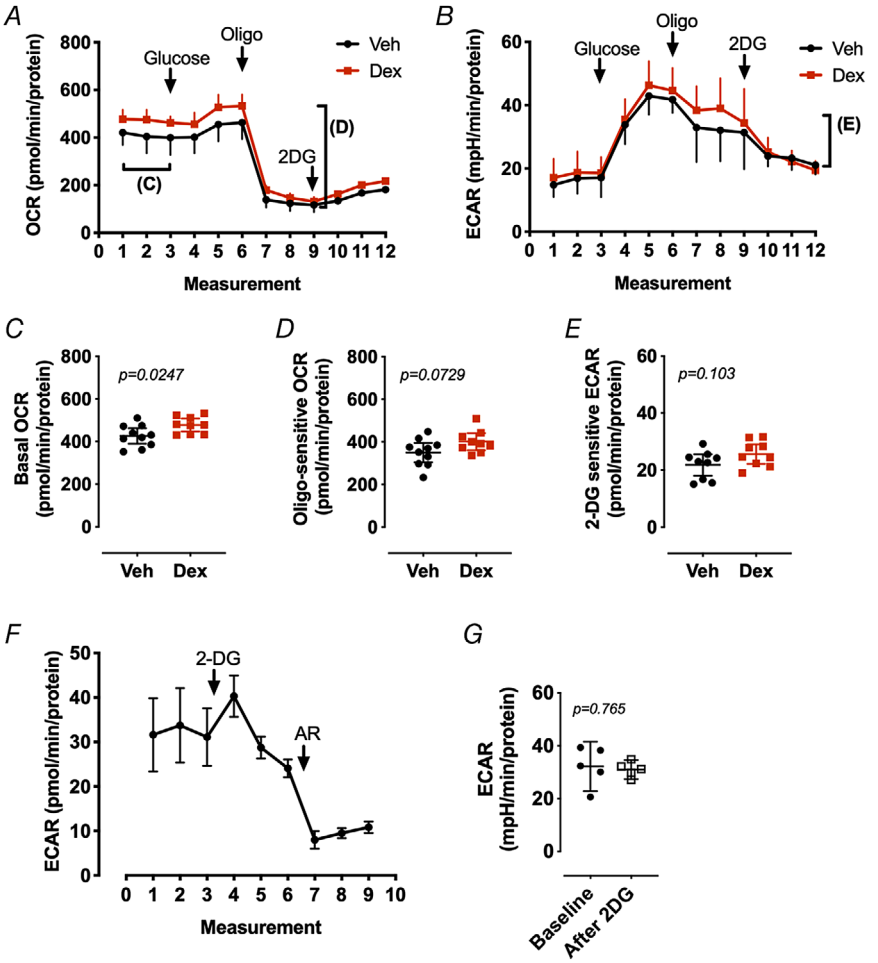

Figure 2. Primary fetal cardiomyocytes rely mainly on mitochondrial oxidation, with little reliance on glycolysis
Primary fetal cardiomyocytes were prepared by digesting pooled E14.5–15.5 C57BL/6J fetal hearts, then cultured for 48 h in complete DMEM culture medium. Cardiomyocytes were treated with dexamethasone (Dex, 1 μM) or vehicle (Veh, 0.01% ethanol). After 24 h, cell culture medium was exchanged for seahorse base medium (supplemented with 1 mM glutamine) and cardiomyocyte metabolism was analysed by extracellular flux assays. After three basal measurements, glucose (10 mM) was added, followed by oligomycin (Oligo, 1.5 μM) and 2-deoxyglucose (2DG, 100 mM). Oxygen consumption rate (OCR, *A*) and extracellular acidification rate (ECAR, *B*) were measured three times over 7.5 min following each addition. Measures used to calculate values shown in panels (*C*–*E*) are indicated with brackets in (*A*) and (*B*). *C*, basal OCR was calculated as the mean of the three basal measurements. *D*, ATP production was estimated as the maximum change in OCR following the addition of oligomycin (oligomycin-sensitive OCR). *E*, glycolysis (2DG-sensitive ECAR) was measured as the maximum change in ECAR following 2DG treatment. For (*F*–*G*), primary fetal cardiomyocytes prepared as above were cultured for 72 h in complete DMEM culture medium. Complete medium was exchanged for seahorse assay medium (supplemented with 10 mM glucose, 1 mM GlutaMAX, 1 mM pyruvate). *F*, ECAR was measured, after three baseline measurements 2DG (100 mM) was added followed by antimycin and rotenone (AR, 2 mM). Three measurements at 2.5 min intervals were recorded after each addition. Measurements were normalized to protein. *G*, the difference between ECAR at baseline and after the glycolysis inhibitor, 2DG was used to estimate the level of glycolysis occurring in these cells. Data are means ± SD and were analysed by Student's *t* tests with $P < 0.05$ as the significance threshold, *P* values below this are indicated in bold; $n = 9$–10 individual wells (*C*–*E*) or $n = 5$ individual wells of primary fetal cardiomyocytes (*G*), pooled from 15–30 fetal hearts and analysed at the same time across one experiment.

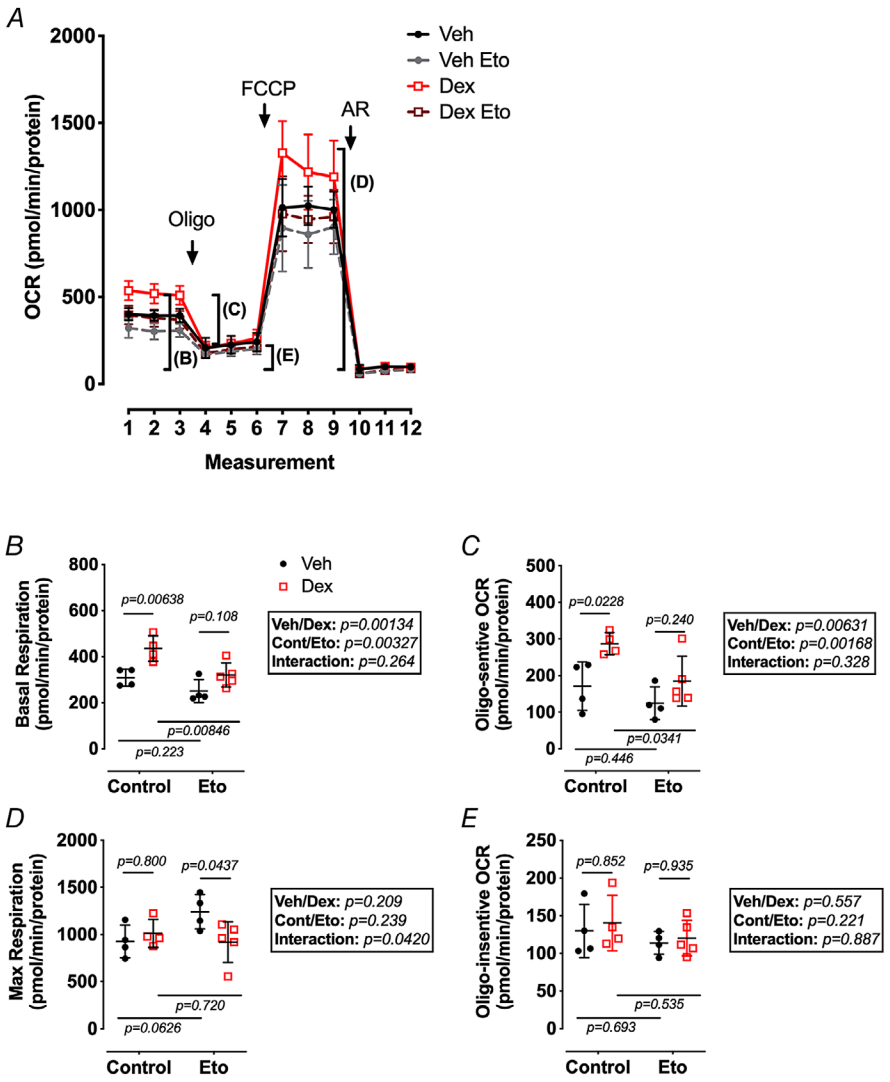

Figure 3. Dexamethasone increases fatty acid oxidation
Primary fetal cardiomyocytes, prepared by digesting pooled E14.5–15.5 C57BL/6J fetal hearts, were cultured for 48 h in complete DMEM culture medium then treated with dexamethasone (Dex, 1 μM, red) or vehicle (Veh, black). After 24 h, medium was exchanged for seahorse assay medium supplemented with 5 mM glucose, 1 mM pyruvate and 0.5 mM carnitine. Cells were treated with etomoxir (Eto, 6 μM) or vehicle (Control) 15 min prior to the addition of BSA-palmitate (100 μM). After a further 15 min incubation, cardiomyocyte metabolism was analysed by extracellular flux assay. After three basal measurements, oligomycin was added (Oligo, 1.5 μM) followed by carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μM) then antimycin and rotenone (AR, 2 μM). Oxygen consumption rate (OCR, *A*) was measured three times over 7.5 min following each drug addition. Measures used to calculate values shown in panels (*B–E*) are indicated with brackets. Non-mitochondrial respiration was estimated as the mean OCR remaining after AR addition. *B*, basal respiration was estimated as the mean of the three basal OCR measurements corrected for non-mitochondrial respiration. *C*, ATP production was estimated as the maximum change in OCR following the addition of oligomycin. *D*, maximum respiration was estimated as the maximum OCR (following FCCP) corrected for non-mitochondrial respiration. *E*, leak respiration was estimated as the mean oligomycin-insensitive OCR corrected for non-mitochondrial respiration. Representative data are from one experiment performed on cardiomyocytes pooled from 15–30 fetuses across $n = 4–5$ individual wells of pooled primary fetal cardiomyocytes. Data are means ± SD and were analysed by two-way ANOVA followed by *post hoc* Sidak's tests with $P < 0.05$ as the significance threshold, *P* values below this indicated in bold. Three samples were excluded due to a technical failure (leakage of AR from the port during basal measurements).

Nr3c1 mRNA, encoding GR, were downregulated ($P = 0.0017$; $n = 4$; Fig. 5*A*), though they recovered by 24 h ($P = 0.137$; $n = 4$, Fig. 5*B*). Pre-treatment with RU486 attenuated the dexamethasone-induced increase in *Ppargc1a*, *Lcad*, *Lipin1* and *Cd36* mRNAs (*P* values for effect of dexamethasone in control *vs.* RU486-treated as follows, *Ppargc1a* - 0.0138 *vs.* 0.158; *Lcad* - 0.00600 *vs.* 0.0186; *Lipin1* - 0.0161 *vs.* 0.115; *Cd36* - 0.00232 *vs.* 0.122; Fig. 6*A*–6*I*, $n = 4$). At E17.5, hearts of $GR^{-/-}$ fetal mice had reduced levels of *Mcad* mRNA ($P = 0.0365$, $n = 5$–6). Although the reduction in levels of *Ppargc1a* mRNA did not achieve significance ($P = 0.210$, $n = 5$–6; Fig. 6*J*) we have previously reported reduced *Ppargc1a* mRNA in hearts of E17.5 $GR^{-/-}$ fetal mice (Rog-Zielinska *et al.* 2013). The failure to reach statistical significance here likely reflects smaller group sizes and overnight matings rather than the time-restricted matings adopted previously.

Glucocorticoids do not cause mitochondrial remodelling in fetal cardiomyocytes

Because there is a wave of mitophagy *in vivo* in the mouse fetal heart that coincides with the peak of fetal corticosterone levels (Rog-Zielinska *et al.* 2013; McWilliams *et al.* 2016), we hypothesized that glucocorticoids may stimulate the mitophagic replacement of fetal mitochondria by adult mitochondria optimized for fatty acid metabolism (Gong *et al.* 2015). *mito*-QC transgenic mice utilize a binary fluorescence system in which a ubiquitously expressed tandem mCherry-GFP tag is directed to mitochondria (McWilliams *et al.* 2016). Under steady-state conditions, the mitochondrial network fluoresces red and green (merged, yellow) in *mito*-QC mice. Upon delivery to lysosomes, the GFP fluorescence, but not that of mCherry, is quenched by the acidic microenvironment. Thus, mitochondria undergoing

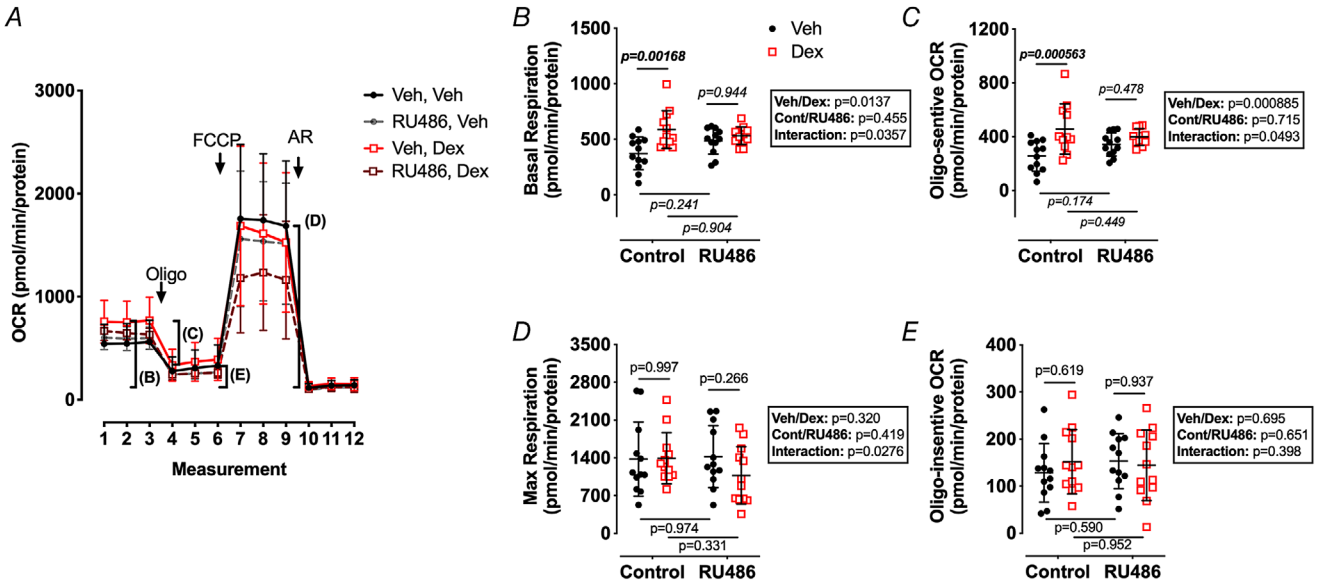

Figure 4. Glucocorticoid receptor (GR) mediates the dexamethasone-induced increase in fatty acid oxidation
Primary fetal cardiomyocytes, prepared by digesting pooled E14.5–E15.5 C57BL/6J fetal hearts were cultured for 48 h before treatment with RU486 (1 μM) or vehicle (control) 30 min prior to addition of dexamethasone (Dex, 1 μM, red) or vehicle (Veh, black). After 24 h, medium was exchanged for seahorse assay medium supplemented with 5 mM glucose, 1 mM pyruvate and 0.5 mM carnitine. Cells were treated with etomoxir (Eto, 6 μM) or vehicle (Control) 15 min prior to addition of BSA-palmitate (100 μM). After 15 min incubation with BSA-palmitate, cardiomyocytes were subjected to extracellular flux assay. After three basal measurements, oligomycin was added (Oligo, 1.5 μM) followed by carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μM) and antimycin and rotenone (AR, 2 μM). Oxygen consumption rate (OCR, *A*) was measured three times over 7.5 min following each drug addition. Measures used to calculate values shown in panels (*B*–*E*) are indicated with brackets. Non-mitochondrial respiration was estimated as the average OCR remaining after AR addition. *B*, basal respiration was estimated as the mean of the three basal OCR measurements corrected for non-mitochondrial respiration. *C*, ATP production was estimated as the maximum change in OCR following addition of oligomycin. *D*, maximum respiration was estimated as the maximum OCR measurement corrected for non-mitochondrial respiration. *E*, leak respiration was estimated as the average oligomycin-insensitive OCR corrected for non-mitochondrial respiration. Data are from pooled cardiomyocytes (n = 12 wells of pooled fetal cardiomyocytes across three different pools) and are means ± SD and analysed by two-way ANOVA followed by *post hoc* Sidak's tests with $P < 0.05$ as the significance threshold, *P* values below this indicated in bold.

mitophagic removal appear as punctate mCherry-only foci. To investigate whether dexamethasone induces mitophagy, primary fetal cardiomyocytes from *mito*-QC mice were treated with dexamethasone, vehicle or a mitophagy-inducing agent, DFP (Allen *et al.* 2013). As expected, DFP stimulated a robust mitophagy response (as visualized by an increase in the number of mCherry-only positive puncta) over the 45 h time course (Fig. 7*A*). In contrast, no significant increase in mitophagy could be seen in cardiomyocytes following a similar time course of dexamethasone treatment ($P = 0.637$ at 45 h, $n = 17$–40; Fig. 7*B*). Thus, under these conditions, dexamethasone is not a potent inducer of mitophagy.

Glucocorticoids have been associated with changes in mitochondrial number and function (Weber *et al.* 2002; Du *et al.* 2009; Lapp *et al.* 2018). Accordingly, we next investigated whether the dexamethasone-induced increase in fatty acid oxidation was associated with an increase in mitochondrial volume and/or number. Following dexamethasone treatment of primary fetal cardiomyocytes for 24 h, mitochondria were labelled with MitoTracker Deep red CM and the mitochondrial network imaged (Fig. 7*C*, 7*D*). There were no differences in mitochondrial volume ($P = 0.287$), length ($P = 0.152$) or width ($P = 0.415$) between dexamethasone and vehicle-treated cardiomyocytes (Fig. 7*E*–7*G*, $n = 17$–40). Furthermore, mitochondrial DNA content, an indirect measurement of mitochondrial number, did not differ between hearts of E17.5 $GR^{-/-}$ mice and their control $GR^{+/+}$ littermates ($P \geq 0.355$, $n = 5$–6; Fig. 7*H*–7*J*. Thus, the glucocorticoid-mediated increase in fatty acid oxidation capacity most likely occurs independently of any change in mitochondrial number or morphology.

In vivo, dexamethasone-induced changes in fatty acid oxidation genes in mouse hearts are developmental stage-dependent

To investigate whether glucocorticoid administration *in vivo* can similarly induce cardiac fatty acid oxidation

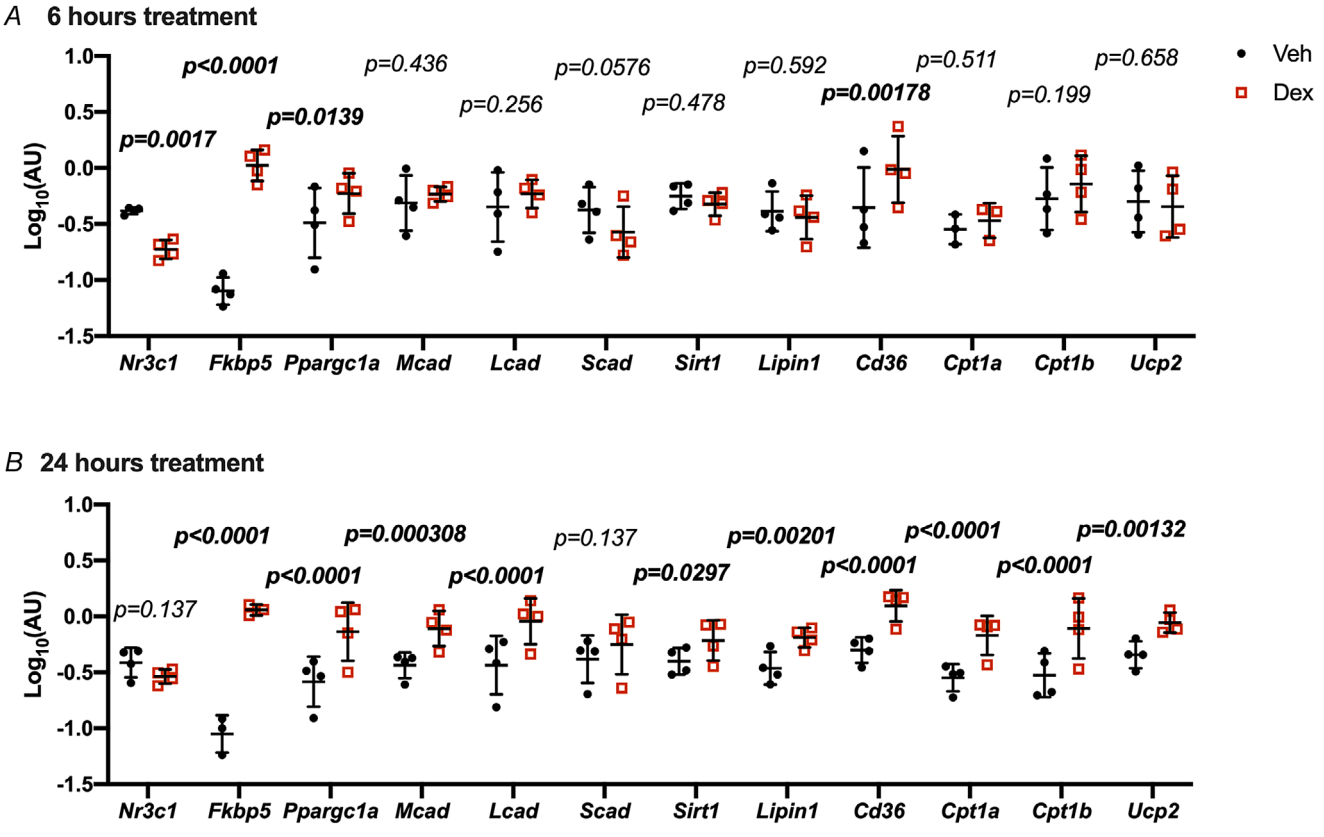

Figure 5. Dexamethasone increases the expression of genes involved in mitochondrial fatty acid oxidation
Primary fetal cardiomyocytes, prepared by digesting pooled E14.5–E15.5 C57BL/6J fetal hearts were cultured for 48 h before treatment with dexamethasone (Dex, 1 μM, red) or vehicle (Veh, black) for (*A*) 6 or (*B*) 24 h. Cardiomyocytes were lysed in TRIzol and RNA isolated for analysis by qRT-PCR relative to *Tbp*, used as internal control. Data are from $n = 4$ independent pools of cardiomyocytes, each from 15–30 fetal hearts, prepared on different days, each data point represents one pool. Data are means ± SD, analysed by paired Fisher's least significant difference tests with $P < 0.05$ as the significance threshold, *P* values below this indicated in bold.

capacity, dexamethasone or vehicle was administered to pregnant dams at E13.5 or E16.5 or to neonatal mice at postnatal day (P)1. Hearts were examined 24 h after injection. E14.5 fetal hearts appeared glucocorticoid-resistant: dexamethasone had no significant effect on any of the mRNAs examined, including the glucocorticoid target, *Fkbp5*, and *Nr3c1* encoding GR itself ($P \geq 0.0944$, $n = 5$; Fig. 8*A*). However, at E17.5, dexamethasone downregulated cardiac *Nr3c1* mRNA levels ($P = 0.035$, $n = 5$; Fig. 8*B*). At the same time, levels of *Ppargc1a* and mRNA encoding enzymes and transporters for fatty acid oxidation (*Mcad, Lcad, Lipin1, Cd36, Cpt1a, CPT1b*) were strongly downregulated (*P* value range from 0.00234 to 0.0331,

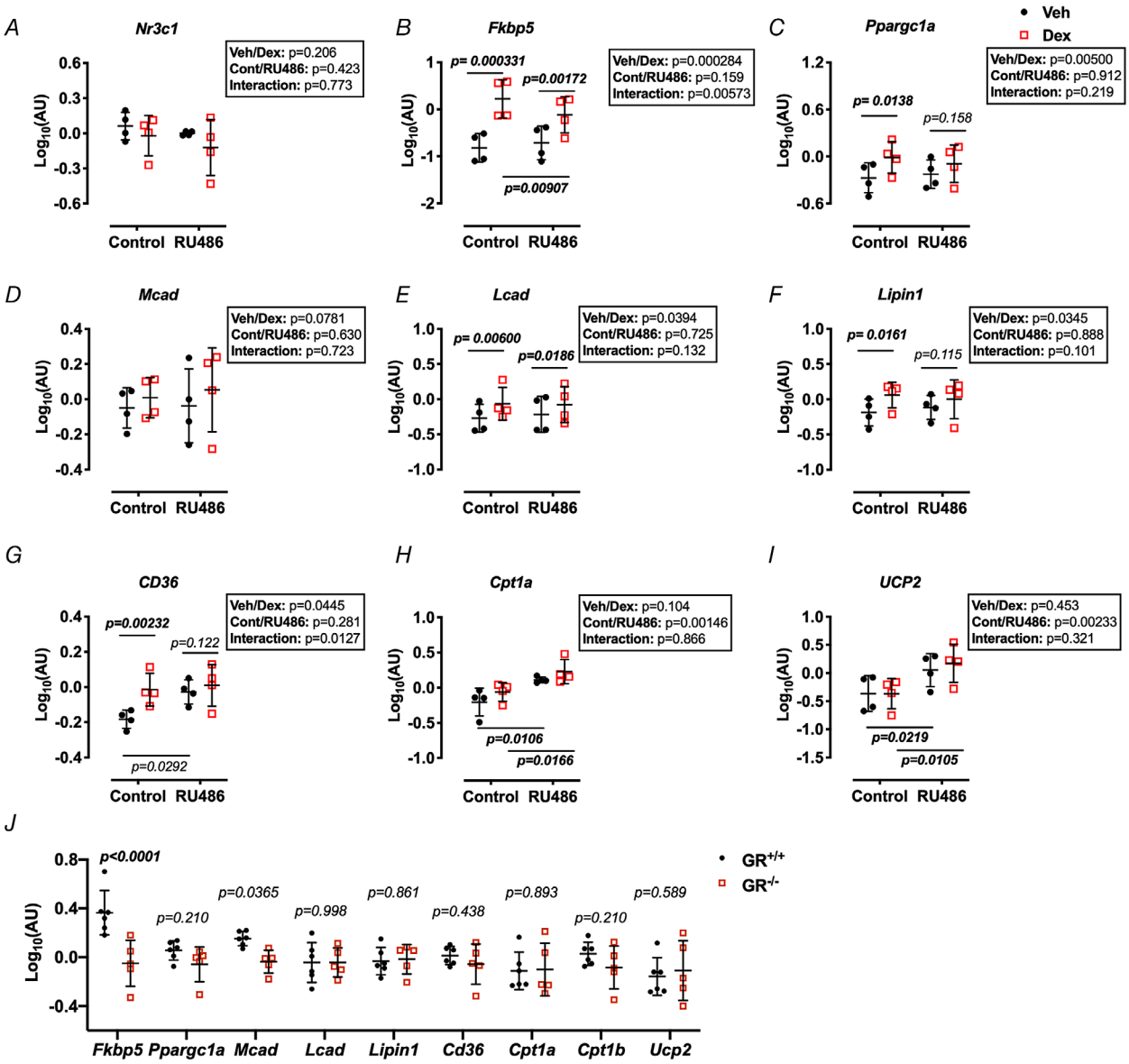

Figure 6. Glucocorticoid receptor (GR) antagonism attenuates the dexamethasone-induced increase in genes involved in fatty acid oxidation and some of these changes are seen in GR$^{-/-}$ fetal hearts at E17.5
(*A–I*) primary fetal cardiomyocytes were prepared by digesting 15–30 pooled C57BL6/J fetal hearts at E14.5–15.5. Cardiomyocytes were cultured for 48 h under standard conditions before being treated with the GR antagonist, RU486 (1 μM) or vehicle (control, 0.01% ethanol) 30 min prior to treatment with dexamethasone (Dex, 1 μM) or vehicle (Veh, 0.01% ethanol). After 24 h, the cardiomyocytes were lysed in TRIzol and RNA isolated for analysis by qRT-PCR. *J*, GR$^{+/+}$ and GR$^{-/-}$ hearts were dissected from E17.5 fetuses and RNA extracted for analysis by qRT-PCR. Data are means ± SD, analysed by repeated measures two-way ANOVA followed by *post hoc* Sidak's tests (*A–I*) or Fisher's least significant difference tests with $P < 0.05$ as the significance threshold, *P* values below this indicated in bold. $n = 4$ independent cardiomyocyte preparations (*A–I*), $n = 5–6$ individual fetus hearts, each from a different litter (*J*).

n = 5), despite a significant, albeit modest, increase in *Fkbp5* mRNA (P = 0.0116, n = 5; Fig. 8*B*). In complete contrast, at P2, *Fkbp5* was strongly induced by dexamethasone (P < 0.0001, n = 7–8), as was *Ppargc1a* (P = 0.0101, n = 7–8) and the fatty acid oxidation genes: *Mcad, Lcad, Cd36, Cpt1b* (P = 0.00246, 0.00511, 0.00195 and 0.0416, respectively, n = 7–8; Fig. 8*C*). Moreover, levels of *Nr3c1* mRNA were unchanged (P= 0.787, n = 7–8; Fig. 8*C*). Thus, the effect of glucocorticoids upon cardiac capacity for fatty acid oxidation reflect the effect upon GR expression itself and its key target gene, *Ppargc1a*. Because this finding was unexpected and has implications for the use of antenatal corticosteroids in preterm birth, we extended our investigation to measure

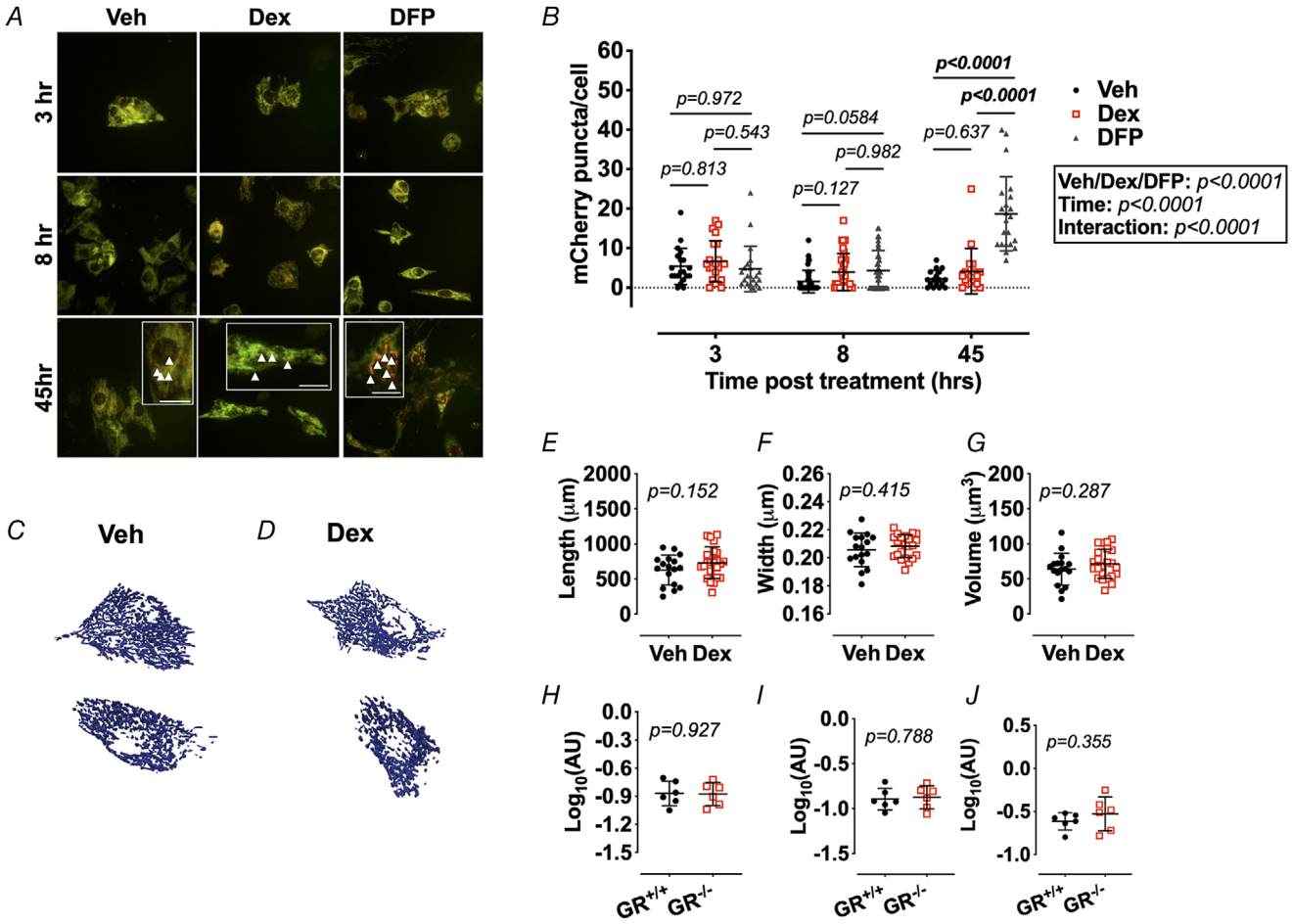

Figure 7. Dexamethasone does not alter mitochondrial morphology or induce mitophagy in primary fetal cardiomyocytes
Primary cardiomyocytes were isolated from 15–30 pooled E14.5–E15.5 *mito*-QC (*A*, *B*) or C57Bl/6J (*C*–*G*) fetal hearts, then cultured for 48 h. *A*, *B*, cardiomyocytes were treated with dexamethasone (Dex, 1 μM), vehicle (Veh) or a mitophagy-inducing agent, deferiprone (DFP, 1 mM) and imaged live, 3, 8 and 45 h later. z stacks of individual cells were obtained using a spinning disk confocal microscope (100× magnification). *A*, representative maximum z projection images are shown: white arrows indicate examples of puncta in magnified panels. Scale bars = 10 and 5 μm for main and high magnification panels, respectively. *B*, puncta were counted manually through z stacks. Data are means ± SD, n = 17–23 individual cells and analysed by two-way ANOVA with *post hoc* Sidak's tests. *C*–*G*, cardiomyocytes were cultured for 48 h then treated with dexamethasone (Dex, 1 μM) or vehicle (Veh) for 24 h. Mitochondria were labelled with MitoTracker Deep Red CM and cardiomyocyte beating stopped with nefidipine (100 mM). z stacks of individual cells were obtained using a spinning disk confocal microscope (100× magnification). Mitochondrial morphology was assessed using MitoGraph software (23, 24) and 3D renderings are shown for vehicle (*C*) and dexamethasone (*D*) treated cardiomyocytes. Parameters included (*E*) length, (*F*) width, and (*G*) total volume. Data are means ± SD, n = 17–40 individual cells in one representative experiment (each data point representing one cell) analysed by Student's t tests. *H*–*J*, mitochondrial DNA was quantified by qPCR of mitochondrially encoded genes: cytochrome c oxidase I (mt-CO1, D), III (mt-CO3, *E*) and NADH dehydrogenase II (mt-ND2, *F*). Absolute values were corrected for genomic *Cebpa* levels. Data are means ± SD and analysed by Student's t tests, n = 6 fetal mouse hearts each from a different litter.

cardiac mRNA encoding GR and PGC-1α in a translational sheep model of preterm birth following antenatal corticosteroid administration that mimics current clinical practice. Indeed, current clinical practice is based on experiments conducted on sheep, and the sheep has been extensively used to investigate the effects of antenatal glucocorticoids on fetal outcomes including long-term outcomes, in adulthood (reviewed in Dickinson *et al.* 2016; Morrison *et al.* 2018). Although dexamethasone is used as an antenatal corticosteroid in the UK and some other countries, celestone, a mix of betamethasone phosphate and betamethasone acetate, is used clinically in the USA, Europe, Australia and New Zealand. We have shown that the slower-release betamethasone acetate, at the same dose as the betamethasone acetate component of celestone, shows equivalent efficacy to the clinical drug in maturing the lungs of fetal rhesus macaques, with the benefit of reduced corticosteroid exposure (Schmidt *et al.* 2019b). Accordingly, both celestone and betamethasone acetate were tested. Preterm lambs delivered at 122 days (term being ~147 days) 48 h after initiating a course of celestone (2 doses, administered 24 h apart) showed reduced expression of *NR3C1* mRNA in both left (P = 0.000903, n = 9 or 11) and right (P = 0.000604, n = 9 or 10) ventricles of the heart (Fig. 9*A*, 9*B*). A single dose of betamethasone acetate (equivalent to just the betamethasone acetate component of celestone) 24 h before delivery also reduced *NR3C1* mRNA levels, though this did not achieve significance in the right ventricle (LV and RV, P = 0.00430 and 0.0750, respectively, n = 10 or 11; Fig. 9*A*, 9*B*). Levels of *PPARGC1* mRNA were significantly reduced in the right ventricle following celestone administration (P = 0.00347, n = 9 or 10), though not in the left ventricle (P = 0.923, n = 9 or 11). Betamethasone acetate alone caused a non-significant reduction in *PPARGC1* mRNA levels in both ventricles (LV, P = 0.655; RV, P = 0.203, n = 10–11; Fig. 9*C*, 9*D*), consistent with its lower potency compared with celestone. Thus, in both mice and sheep, antenatal corticosteroid administration may interfere with the normal maturation of the mid- to late gestation heart by downregulating GR.

Discussion

Here, we find that mouse fetal cardiomyocytes *in vitro* use mainly oxidative phosphorylation to generate ATP when glucose is provided as substrate. This supports the view that metabolism has switched from anaerobic glycolysis to aerobic mitochondrial respiration by the

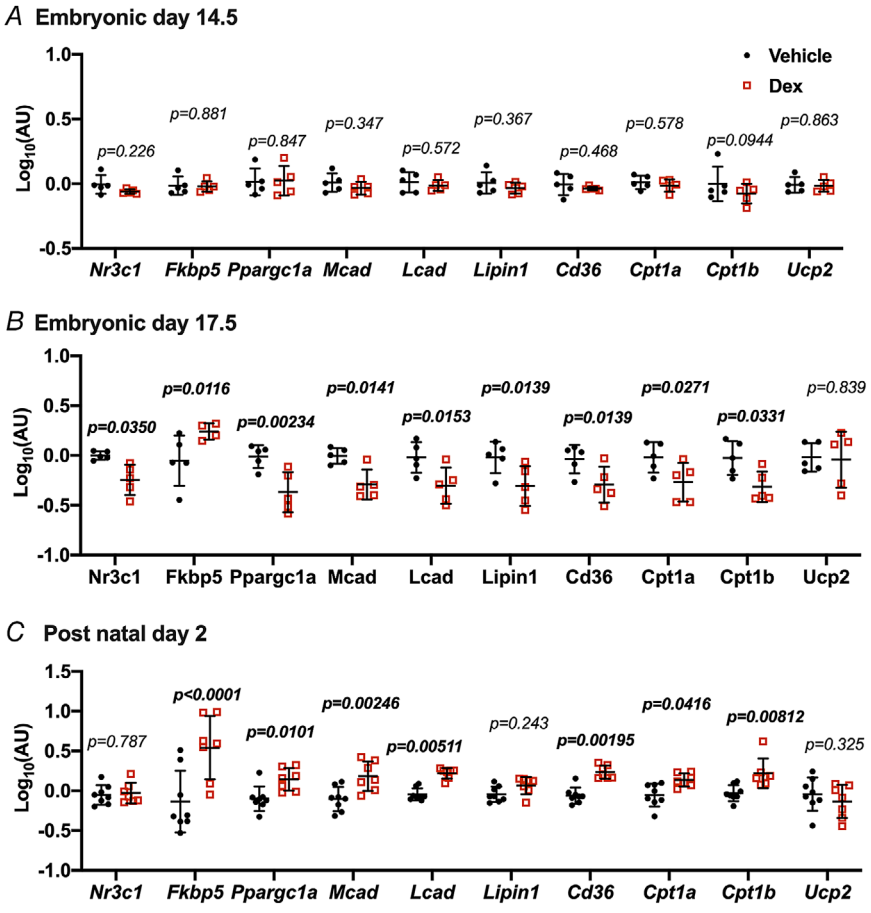

Figure 8. Dexamethasone regulates cardiac mitochondrial fatty acid oxidation in perinatal mice *in vivo*
Pregnant C57Bl/6J dams were injected (I.P.) with 0.5 mg/kg dexamethasone or vehicle at E13.5 (*A*) or E16.5 (*B*). C57Bl/6J neonates (*C*) were injected on P1 with dexamethasone (0.5 mg/kg) or vehicle. After 24 h hearts were excised and analysed by qRT-PCR for genes involved in fatty acid oxidation. For (*A*, *B*) n = 5 litters; each data point represents one litter, with one or two animals per litter analysed (where two animals from a litter were analysed, the mean is shown). One *Fkbp5* data point in the dexamethasone group at E17.5 was identified as an outlier using Grubbs' test and excluded prior to further analysis. For (*C*), n = 7–8 neonates with one animal per litter analysed; each data point represents one animal. Data are means ± SD and analysed by unpaired Fisher's least significant difference tests, with $P < 0.05$ as the significance threshold, P values below this are indicated in bold.

end of the embryonic period at E14.5 in mice (reviewed Porter *et al.* 2011). Although glucocorticoids increase basal mitochondrial respiration (confirming previous findings (Rog-Zielinska *et al.* 2015)), they have no effect on glycolysis, ruling out a glucocorticoid-promoted switch from glycolysis to oxidative metabolism. Our data do not support a glucocorticoid-mediated increase in mitochondrial number or change in morphology to account for the increase in basal respiration. Instead, our data suggest that glucocorticoid action in the fetal heart promotes mitochondrial ATP-generating capacity, in line with the maturational effects of glucocorticoids.

As well as increasing basal mitochondrial respiration with glucose as substrate, dexamethasone treatment of fetal cardiomyocytes *in vitro* increases mitochondrial fatty acid oxidation. It has been suggested that mitophagy is required to replace the mitochondrial network in perinatal cardiomyocytes with mitochondria optimized for fatty acid oxidation (Gong *et al.* 2015). However, our data suggest that an increase in fatty acid oxidation can occur in fetal cardiomyocytes without substantial mitophagy. Moreover, they are consistent with the notion that mitochondrial remodelling occurs in cardiomyocytes prior to our cell isolations at ∼E15. Mitochondrial phenotype in the embryonic heart changes considerably between E9.5 and E13.5, compatible with mitochondrial remodelling by mitophagy being required for the switch in cardiomyocyte reliance from anaerobic glycolysis to aerobic respiration. By E13.5, the network is interconnected and spans the cell, more closely resembling that in late fetal cardiomyocytes (Porter *et al.* 2011). Our conclusions differ from a recent report that suggests that dexamethasone promotes mitophagy in mouse embryonic stem cell-derived cardiomyocytes through Parkin (Zhou *et al.* 2020). We note that in those experiments, detection of lysosomes (using lysotracker) was only possible in dexamethasone-treated cells (Zhou *et al.* 2020) making interpretation of the mitophagy findings difficult. Nevertheless, dexamethasone did not affect mitochondrial morphology in mouse embryonic stem cell-derived cardiomyocytes (Zhou *et al.* 2020), consistent with our findings here. Our data reported here clearly show that although glucocorticoid treatment of primary mouse fetal cardiomyocytes increases fatty acid oxidation, it does not induce widespread mitophagy.

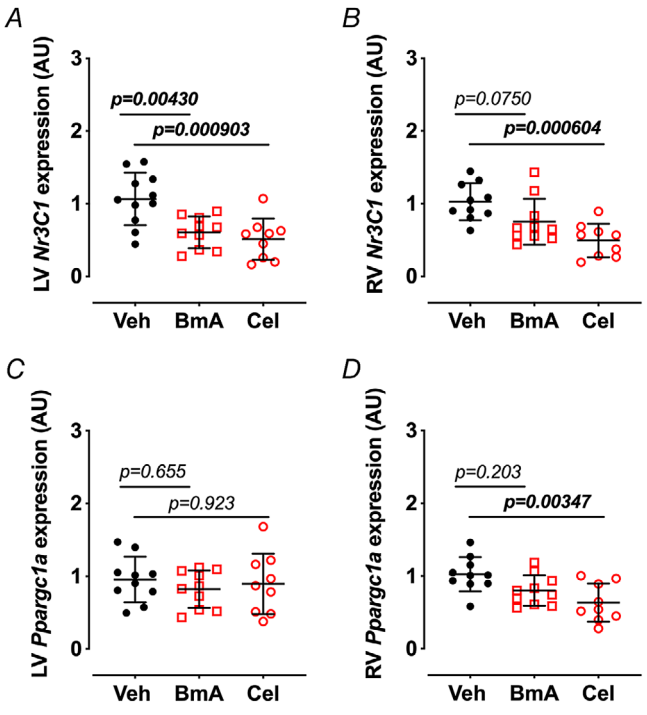

Figure 9. Cardiac glucocorticoid receptor (GR) and PGC-1α expression are reduced following treatment with antenatal corticosteroids in a sheep model of preterm birth
Lambs were delivered preterm at 122 ± 1 days (term being ∼147 days), 48 h after initiating a course of celestone (Cel: 2 doses, 24 h apart) or 24 h after a single dose of betamethasone acetate (BmA). *A*, *B*, mRNA encoding GR (*NR3C1*) and (*C*, *D*) PGC-1α (*PPARGC1A*) were measured by qPCR in the left ventricle (LV) and right ventricle (RV). Data are means ± SD, data were first analysed with Grubbs' test and one outlier (in the LV vehicle group) was detected and removed from the analysis. Subsequently the data were analysed by ordinary one way ANOVA with *post hoc* Tukey's tests with $P < 0.05$ as the significance threshold, *P* values below this are indicated in bold. For RV: Veh $n = 10$, BmA $n = 10$, Cel $n = 9$. For LV: Veh $n = 11$, BmA $n = 10$, Cel $n = 9$.

Dexamethasone treatment of fetal cardiomyocytes *in vitro* increases expression of genes related to fatty acid oxidation. Fatty acid oxidation genes themselves are not primary targets of GR in mouse fetal cardiomyocytes (Rog-Zielinska *et al.* 2015) and are likely indirectly regulated by master regulators of fatty acid oxidation including CEBPβ, PPARα and PGC-1α, which we have previously shown are primary GR targets in fetal cardiomyocytes (Rog-Zielinska *et al.* 2015). The induction of PPARα by glucocorticoids provides a plausible mechanism by which dexamethasone increases genes in the mitochondrial fatty acid oxidation pathway: PPARα target genes include those responsible for almost every step of mitochondrial fatty acid oxidation as well as fatty acid import into mitochondria (Rakhshandehroo *et al.* 2010). Other mechanisms may contribute to the increase in fatty acid oxidation resulting from dexamethasone treatment of fetal cardiomyocytes *in vitro*. We have previously shown *Atp5g2*, encoding a subunit of the F0 component (the proton channel) of mitochondrial ATP synthase to be a direct target of GR and rapidly induced by dexamethasone in fetal cardiomyocytes (Rog-Zielinska *et al.* 2015). This suggests that glucocorticoids have the potential to directly alter the capacity for ATP generation through oxidative phosphorylation. Complexes I to IV are not direct targets of GR, so if their expression is altered by dexamethasone,

this is likely indirect. CPT-1 is not a direct target of GR in fetal cardiomyocytes (Rog-Zielinska *et al*. 2015). However, CD36, a cell surface receptor also known as fatty acid translocase is a direct target of GR and is increased in the current study. This is likely to increase capacity of cardiomyocytes to import fatty acids, available for their mitochondrial oxidation. In sum, our data *in vitro* provide proof-of-principle evidence that glucocorticoids can increase capacity for mitochondrial fatty acid oxidation in fetal heart maturation.

Fatty acid oxidation dramatically increases around the end of the first postnatal week in mouse heart (Lopaschuk & Jaswal, 2010). However, in preparation for postnatal life, fatty acid oxidation has already begun to occur in the late gestation fetal heart (Lopaschuk & Jaswal, 2010; Porter *et al*. 2011). In sheep, there is an increase in cardiac expression of genes related to fatty acid oxidation between late gestation and term that continues after birth (Richards *et al*. 2015). *In silico* transcription factor analysis suggests that this is, at least in part, GR-mediated (Richards *et al*. 2015). The lower level of *Mcad* mRNA in hearts of GR knockout fetuses at E17.5, a stage when endogenous glucocorticoid levels have increased, supports a role for GR in the normal late gestation increase in cardiac expression of genes required for fatty acid oxidation. Thus, *endogenous* glucocorticoid action, via GR, may contribute to the normal rise in fatty acid oxidation capability as the fetus approaches term. This is likely to be important to meet the increase in cardiac energy demand after birth, consistent with the ergogenic effects of glucocorticoids (Addison, 1855; Morrison-Nozik *et al*. 2015) and the vital role of the late gestation increase in glucocorticoids to prepare for life after birth.

Crucially, our data illustrate how *exogenous* glucocorticoid may interfere with the normal maturation of energy metabolism in the late gestation fetal heart – and potentially other aspects of normal cardiomyocyte maturation – by downregulating the capacity to respond to glucocorticoids. Although dexamethasone administration *in vivo* in neonates markedly induces cardiac expression of genes involved in the fatty acid oxidation pathway, similar to fetal cardiomyocytes *in vitro*, dexamethasone has the *opposite* effect in the late gestation mouse fetal heart. This striking difference between the *in vitro* response of fetal cardiomyocytes and the *in vivo* response in the late gestation heart plausibly reflects different responses of GR itself to treatment. In the E17.5 fetal heart, the reduction in *Nr3c1* mRNA following dexamethasone treatment suggests the decrease in the mitochondrial fatty acid oxidation pathway reflects downregulation of glucocorticoid signalling *per se*. We recently reported similar downregulation of GR expression in fetal heart as well as reduced endogenous fetal corticosterone levels following dexamethasone treatment (via drinking water) between E12.5 and E15.5 (Agnew *et al*. 2019). This was associated with a transient alteration in fetal diastolic heart function (Agnew *et al*. 2019). Whilst dexamethasone also downregulates *Nr3c1* mRNA in fetal cardiomyocytes *in vitro*, this is transient with recovery of *Nr3c1* mRNA expression by 24 h. In the neonatal heart, if *Nr3c1* mRNA is transiently downregulated by dexamethasone, it has recovered within 24 h. Dynamic and differential autoregulation of GR, previously described for adult tissues (Kalinyak *et al*. 1987; Spencer *et al*. 1991; Freeman *et al*. 2004), may contribute to the complex and context-dependent effects of perinatal glucocorticoid administration. Previous studies have examined the effect of antenatal glucocorticoids in rodents and reported contradictory findings (reviewed, Rog-Zielinska *et al*. 2014). Although this likely involves differences in the nature of the administered glucocorticoid, for example, cortisol or corticosterone *vs*. dexamethasone, our findings support dependence on the timing as well. Importantly, they illustrate that during particular gestational windows, exogenous glucocorticoids may interfere with normal heart maturation. Indeed, maturation of the rat heart is delayed after prenatal treatment with dexamethasone (Torres *et al*. 1997). GR expression was not examined in that study. Investigations in sheep have also highlighted adverse effects of antenatal glucocorticoid exposure on cardiac energy metabolism. Maternal hypercortisolaemia reduces fetal cardiac mitochondrial number and oxidative metabolism at term, associated with fetal ECG abnormalities, an inability to maintain fetal aortic pressure and heart rate during labour and a dramatic increase in perinatal death (Antolic *et al*. 2018). Again, whether GR expression was affected was not reported. In our experiments, cardiac expression of GR in preterm neonates was reduced following exogenous glucocorticoid administration in late gestation in sheep, similar to the downregulation that occurred in fetal mice. This likely limits the capacity to respond to glucocorticoids in sheep heart. Despite considerable differences in the physiology of gestation between mice and sheep (Dickinson *et al*. 2016) and given that the lambs were born (albeit for less than 40 min) whereas the mice were fetuses, this suggests downregulation of GR may be a common response to antenatal corticosteroids. Downregulation of GR and ability to respond to glucocorticoids may be a mechanism that contributes to the well-known association between early-life exposure to synthetic glucocorticoids and chronic adult cardiovascular disease (Fowden *et al*. 1998; Rog-Zielinska *et al*. 2014). Whilst effects on hypothalamic-pituitary-adrenal axis activity and blood pressure may play a role in the programming of cardiovascular disease risk, our data support direct effects on cardiomyocytes as well. Recent evidence suggesting that prenatal dexamethasone exposure alters the trajectory of perinatal heart maturation through alterations in cardiomyocyte mitochondrial function is

also consistent with direct actions in heart (Peng *et al.* 2018).

Differential mRNA stability may explain some of the complex effects of glucocorticoids on downstream genes. PGC-1α is essential for efficient and maximal fatty acid oxidation and ATP production in cardiomyocytes (Arany *et al.* 2005; Lehman *et al.* 2008). *Ppargc1a* mRNA has a short half-life, being less than 30 min in rat skeletal muscle extracts. This is further decreased with chronic muscle stimulation (Lai *et al.* 2010). Consistent with a short half-life, we have previously shown that blocking new protein synthesis with cycloheximide increases *Ppargc1a* mRNA levels in fetal cardiomyocytes (Rog-Zielinska *et al.* 2015), suggesting it is actively degraded. Glucocorticoid treatment rapidly increases levels of *Ppargc1a* mRNA in fetal heart *in vivo* and in fetal cardiomyocytes *in vitro* (Rog-Zielinska *et al.* 2015). Moreover, dexamethasone 'super-induces' *Ppargc1a* in the presence of cycloheximide (Rog-Zielinska *et al.* 2015), again consistent with a rapid turnover of *Ppargc1a* mRNA. A very short half-life and a need for activated GR to continually enhance transcription of *Ppargc1a* mRNA may explain the close association between *Nr3c1* and *Ppargc1a* mRNA in both fetal heart and mouse primary cardiomyocytes that we saw here. By contrast, *in vitro* in fetal cardiomyocytes, *Fkbp5* mRNA was upregulated by dexamethasone, despite transient down-regulation of GR at 6 h. *Fkbp5* mRNA may be a stable readout of early GR activation whereas *Ppargc1a* mRNA correlates with *Nr3c1* mRNA at any particular time. Plausibly, antentatal corticosteroids may disrupt the normal maturation of energy metabolism in the human fetal heart, in part by downregulating PGC-1α. In our sheep model, which closely mirrors clinical practice, both *Nr3c1* and *Ppargc1a* were downregulated in fetal heart by antenatal corticosteroids. Whether this is transient, and both later recover to control levels merits further investigation. Nevertheless, this suggests that clinical administration of antenatal corticosteroids in mid- and late gestation may interfere with normal heart maturation.

The E13.5 fetal heart appears resistant to dexamethasone. The reason for this is unclear, but it has implications for clinical practice. In humans, the reduction in fetal heart rate variability (a clinical marker of fetal hypoxia/poor outcomes) 2 to 3 days after maternal administration of glucocorticoids is greater in fetuses of >30 weeks gestation than those at <30 weeks (Mulder *et al.* 2009), consistent with gestation stage-dependent effects of antenatal glucocorticoids. The greater sensitivity to the haemodynamic effects of glucocorticoids coincides with an increase in fetal cortisol synthesis, from $\sim$30 weeks gestation (Hillman *et al.* 2012). In mice, adrenal steroidogenesis initiates at E14.5 (Michelsohn & Anderson, 1992). This raises the possibility that the fetus is glucocorticoid-resistant prior to the gestational increase in fetal glucocorticoid levels. Conceivably, this might reflect mistiming relative to the ontogeny of thyroid hormone production discussed above. It is intriguing to speculate that glucocorticoids may act in concert with thyroid hormones to increase cardiac mitochondrial oxidative capacity in preparation for birth. In skeletal muscle of fetal sheep, mitochondrial oxidative capacity positively correlates with plasma thyroid hormone (T_3 and T_4) and cortisol levels (Davies *et al.* 2020). The increase in thyroid hormones underlies the gestational increase in mitochondrial density in ovine fetal skeletal muscle, whereas it has no effect on expression of PGC-1α (Davies *et al.* 2020). We have previously shown that the *Dio2* gene encoding the enzyme responsible for converting T_4 to active T_3, is a glucocorticoid target gene in fetal cardiomyocytes (Rog-Zielinska *et al.* 2015). This supports the interdependence of glucocorticoid and thyroid hormone actions in maturation of fetal organs and tissues and raises the possibility that mistimed elevations in plasma glucocorticoid levels might occur at inappropriate (for stage) thyroid hormone levels potentially manifesting as glucocorticoid resistance. A permissive role for T_3 for dexamethasone to mediate effects on the electrophysiology of human pluripotent stem cell-derived cardiomyocytes has been described (Birket *et al.* 2015). Whether the fetus is glucocorticoid-resistant prior to initiation of adrenal steroidogenesis merits future investigation, given widespread expression of GR in the mouse fetus prior to E14.5 (Rog-Zielinska *et al.* 2013). It is interesting to note that although dexamethasone increases calcium handling and contraction force in human embryonic stem cell-derived cardiomyocytes (Kosmidis *et al.* 2015), human induced pluripotent stem cell-derived cardiomyocytes (roughly corresponding to first trimester human fetal cardiomyocytes (van den Berg *et al.* 2015)) do not respond to dexamethasone alone (requiring T_3), possibly because they lack a competence factor (Birket *et al.* 2015). The acquisition of competence to respond to glucocorticoids as well as the autoregulation of GR itself may therefore be developmentally regulated and may differ between cell types. Understanding how this contributes to the maturational effects of glucocorticoids upon fetal organs and tissues will be vital to optimize antenatal corticosteroid therapy in the future, to limit possible harm and maximize benefit.

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Additional information

Data availability statement

The data that support the findings of this study are available from the first and/or corresponding author upon reasonable request.

Competing interests

None of the authors have a competing financial or other conflict of interest.

Author contributions

Experiments were performed and data acquired by the following: in Edinburgh, UK: K.E.C. laboratory – J.R.I., C.B., H.U., E.A.R.-Z., E.P., E.J.A.; N.M.M. laboratory – J.R.I., R.N.C.; S.J.S. laboratory – L.H., C.N.; in Dundee, UK, I.G.G. laboratory – J.-F.Z., J.R.I. and in Perth, Western Australia, M.W.K. laboratory – M.W.K., S.J.S., J.R.I., K.E.C., I.G.G., N.M.M., C.W., S.J.S. were involved in the conception and/or design of the work. All authors were involved in drafting the work or revising it critically for important intellectual content and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and qualify for authorship. All those who qualify for authorship are listed.

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antenatal corticosteroids, cardiomyocytes, early-life programming, glucocorticoid, heart, preterm birth

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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Supplementary Table 1
Statistical Summary Document