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Glucocorticoids promote structural and functional maturation of foetal cardiomyocytes: a role for PGC-1α

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Glucocorticoid levels rise dramatically in late gestation to mature foetal organs in readiness for postnatal life. Immature heart function may compromise survival. Cardiomyocyte glucocorticoid receptor (GR) is required for the structural and functional maturation of the foetal heart *in vivo*, yet the molecular mechanisms are largely unknown. Here we asked if GR activation in foetal cardiomyocytes *in vitro* elicits similar maturational changes. We show that physiologically relevant glucocorticoid levels improve contractility of primary-mouse-foetal cardiomyocytes, promote Z-disc assembly and the appearance of mature myofibrils, and increase mitochondrial activity. Genes induced *in vitro* mimic those induced *in vivo* and include PGC-1α, a critical regulator of cardiac mitochondrial capacity. SiRNA-mediated abrogation of the glucocorticoid induction of PGC-1α *in vitro* abolished the effect of glucocorticoid on myofibril structure and mitochondrial oxygen consumption. Using RNA sequencing we identified a number of transcriptional regulators, including PGC-1α, induced as primary targets of GR in foetal cardiomyocytes. These data demonstrate that PGC-1α is a key mediator of glucocorticoid-induced maturation of foetal cardiomyocyte structure and identify other candidate transcriptional regulators that may play critical roles in the transition of the foetal to neonatal heart.

Maturation of foetal tissues in readiness for the transition from foetal to postnatal life is dependent on the dramatic rise in glucocorticoid levels shortly before birth.[1–3] Hence, potent-synthetic glucocorticoids are routinely administered to preterm babies or foetuses at risk of premature birth, to improve neonatal survival.[4] In addition to the beneficial effects of glucocorticoids on lung maturation, we have recently shown that glucocorticoids are vital to mature foetal-heart structure and function. Endogenous glucocorticoids act *via* glucocorticoid receptor (GR) in foetal cardiomyocytes/vascular smooth muscle to promote myofibril assembly and organisation, thus advancing systolic function and cardiac ultrastructural organisation *in vivo*.[5] These maturational effects are associated with mRNA changes in the foetal heart indicative of improved contractile function, calcium handling and energy metabolism. However, the genes directly under GR control in cardiomyocytes responsible for these vital changes are unknown.

In foetal mice, levels of the endogenous glucocorticoid, corticosterone, are negligible in heart at embryonic day (E) 14.5, but rise rapidly once adrenal corticosterone synthesis begins at E15,[6] peaking at E16.5.[5,7] Consistent with this, GR only becomes localised to the nucleus in mouse cardiomyocytes at E15.5, suggesting the key events initiated by GR

activation occur over 24 h or less. Although several studies have reported glucocorticoid-induced mRNA changes in 24 h in primary rodent neonatal cardiomyocytes, foetal cardiomyocytes are less mature than neonatal.[8] Also, unlike neonatal cardiomyocytes, foetal cardiomyocytes have not had prior exposure to high levels of glucocorticoids *in utero*, which can alter the response to subsequent glucocorticoid exposure,[9] itself highly cell and context dependent.[10,11] Moreover, no previous studies in cardiomyocytes have examined the primary events initiated by GR activation. Here, we used primary-mouse-foetal cardiomyocytes to demonstrate direct actions of glucocorticoids in recapitulating the structural, functional and biochemical consequences of GR activation *in vivo*. Moreover, we have identified genes directly regulated by GR and show a critical role for induction of the transcriptional coactivator, PGC-1α in the structural maturation of foetal cardiomyocytes induced by glucocorticoid treatment *in vitro*.

Results

Primary mouse foetal cardiomyocytes are glucocorticoid responsive. We first established that mouse foetal cardiomyocytes in culture contain functional GR. Immunofluorescence

confirmed nuclear translocation of GR within 1 h of addition of physiological glucocorticoid, corticosterone, or the potent-synthetic glucocorticoid, dexamethasone, to cardiomyocytes isolated from E14.5–E16.5 C57BL/6 mouse foetal hearts (Supplementary Figure 1B). Levels of *Dusp1* and *Fkbp5* mRNAs, known GR target genes[12,13] were increased following glucocorticoid treatment (Supplementary Figure 1C).

Corticosterone promotes sarcomeric organisation and myofibril assembly in foetal cardiomyocytes. Having confirmed a response to glucocorticoids, we asked whether glucocorticoid treatment *in vitro* can mimic the improvement in myofibrillar structure evoked by GR activation *in vivo* in cardiomyocytes.[5] Primary foetal cardiomyocytes were treated with 100 nM corticosterone, approximating levels in the wild-type late gestation (E16.5–E17.5) foetal heart, for 24 h. As well as inducing the formation of troponin T-associated *α*-smooth muscle actin stress fibres in primary foetal cardiomyocytes (Supplementary Figure 1D), corticosterone dramatically altered myofibril organisation, visualised by staining of *α*-actinin (Figure 1a), a constituent of myofibrillar Z-discs. Though Z-disc structures were observed in untreated cells, they became better defined, aligned, and regularly distributed throughout cardiomyocytes following the corticosterone treatment (Figure 1a). The overall appearance of myofibrillar structure was improved, and the sarcomere length (measured as the distance between neighbouring Z-discs) and Z-disc width were increased following the corticosterone treatment (Figure 1b). Pre-treatment with the GR antagonist, RU38486 (RU486), abolished the effect of corticosterone on myofibrillar structure, demonstrating its dependence on GR (Figures 1a and b).

Transmission electron microscopy of sections through primary foetal cardiomyocytes showed a striking increase in contractile fibres in corticosterone-treated cardiomyocytes with clearly delineated myofibrils often associated with well-defined Z-discs (Figure 1c). In contrast, very few Z-discs were apparent in untreated cells and although some fibrillar material was present, it was predominantly disorganised and scattered throughout the cell.

Corticosterone improves contractility of foetal cardiomyocytes. We next asked, whether the improved myofibrillar structure is associated with improved function by measuring the effect of corticosterone on the contractile properties of primary foetal cardiomyocytes using soft-video edge detection. Treatment with 100 nM corticosterone for 24 h did not affect the frequency of spontaneous contractions (Figure 2a; control 35.1 ± 1.3 beats/min *versus* corticosterone 39.0 ± 1.8 beats/min; $n = 38$ cells) but markedly increased contraction amplitude (Figures 2b and c). Corticosterone also decreased contraction and relaxation times (bottom-to-peak and peak-to-bottom time, respectively; Figure 2d) and improved the relaxation kinetics, measured as the time taken to reach 90% relaxation (Figure 2e). Thus, structural improvements elicited by corticosterone are associated with improved contractile function.

Glucocorticoids *via* GR regulate candidate gene expression in cardiomyocytes. To establish that the transcriptional
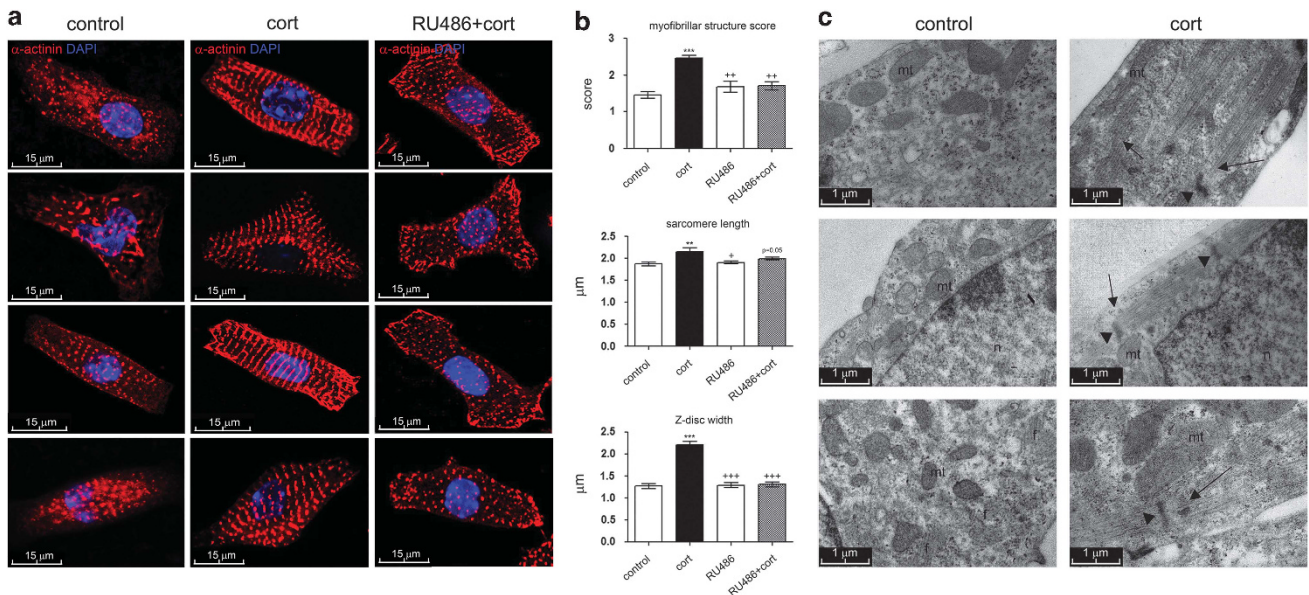

Figure 1 Corticosterone promotes myofibril maturation in foetal cardiomyocytes in a GR-dependent manner. (**a**) Representative images of control (untreated) primary foetal C57BL/6 cardiomyocytes (left panels), or following 100 nM corticosterone (cort) for 24 h (centre panels). To block GR-mediated effects, cells were pre-treated with 1 *μ*M RU38486 (RU486) for 30 min prior to addition of 100 nM corticosterone for 24 h (RU486+cort; right panels). Cells were stained with *α*-actinin (red, Z-discs) and DAPI (blue, nuclei). (**b**) Quantification of the effect of 100 nM cort on myofibrillar structure (top graph, see Materials and Methods for details of the scoring system), sarcomere length (middle graph), and Z-structure/Z-disc width (bottom graph). Data were analysed by one-way ANOVA with Bonferroni's *post-hoc* test; **$P < 0.01$, ***$P < 0.001$ *versus* control; +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ *versus* cort; $n = 50$–80 replicates over three independent experiments. (**c**) Representative transmission electron micrographs of sections of control primary foetal C57BL/6 mouse cardiomyocytes (left panels), or following 100 nM corticosterone (cort) for 24 h (right panels); arrows, myofibrils; arrowheads, Z discs; mt, mitochondria; f, fibres; n, nuclei. Images representative of $n = 10$ cells
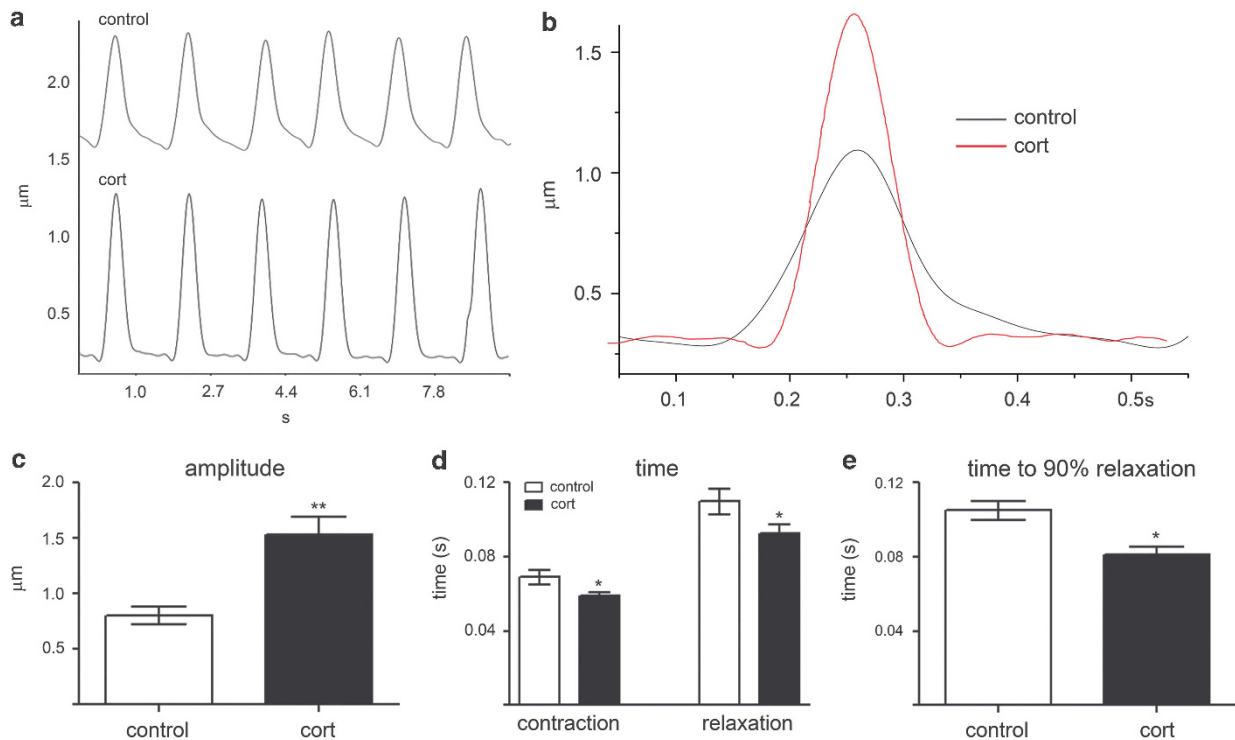
Figure 2 Corticosterone treatment improves the contraction-relaxation of foetal cardiomyocytes. Cells were treated with 100 nM corticosterone for 24 h prior to edge-detection analysis. (**a**, **b**) Representative edge-detection traces showing single contraction (upwards)/relaxation (downwards) events for untreated (control/black) and corticosterone-treated (cort/red) cells. Corticosterone increased the amplitude of contraction (**c**), measured as the distance travelled by the cardiomyocyte edge during the shortening phase, and (**d**) decreased the time of contraction and relaxation as well as (**e**) decreased time taken to reach 90% relaxation. Data were analysed by Student's *t*-test; *$P < 0.05$, **$P < 0.01$; $n = 38$ cells

response of primary foetal cardiomyocytes to glucocorticoids resembles that of the foetal heart, we measured mRNAs that we have previously shown to be altered in heart by glucocorticoid action *in vivo*.[5] *Tsc22d3* (encoding glucocorticoid-induced leucine zipper) represents a classical glucocorticoid-regulated gene, with *Myh6* (encoding myosin heavy chain-*α*; MyHC*α*) and *Ppargc1a* (encoding PPAR*γ* coactivator-1*α*; PGC-1*α*) representative of structural and metabolic genes, respectively. *Nppa* (encoding atrial natriuretic peptide) is known to be glucocorticoid regulated in neonatal rat cardiomyocytes.[8,14] Corticosterone activates GR, but can also activate mineralocorticoid receptor (MR) in the absence of 11*β*-hydroxysteroid dehydrogenase-2.[15,16] We therefore compared the effect of corticosterone with that of dexamethasone, which poorly activates MR.[16] Dexamethasone induced all four mRNAs in a dose-dependent manner over 24 h (Figures 3a–d). Similarly, 100–200 nM corticosterone, approximating concentrations in the late-gestation mouse heart, increased *Tsc22d3*, *Nppa,* and *Ppargc1a* mRNA levels (Figures 3e–h). The modest increase in *Myh6* mRNA levels with 100 nM corticosterone was not observed with the higher dose of 200 nM corticosterone. Glucocorticoid treatment also induced genes involved in calcium handling: *Cacna1c*, *Ryr2*, *Atp2a2*, and *Slc8a1*, encoding respectively, Ca$_v$1.2 (voltage-gated Ca^{2+} channel), ryanodine receptor-2, SERCA2a (sarco/endoplasmic reticulum Ca^{2+} ATP-ase 2a) and NCX1 (Na$^+$/Ca^{2+} exchanger; Supplementary Figures 2A and D), consistent with effects of endogenous GR activation in foetal heart *in vivo*.[5]

RU486 antagonism of GR blocked the glucocorticoid induction of *Tsc22d3*, *Myh6*, *Nppa*, and *Ppargc1a* mRNAs (Figure 4) whereas the MR antagonist spironolactone had no effect (Supplementary Figures 2E and H). Similarly, siRNA mediated knock-down of GR (80% and 35% reduction in GR mRNA and protein levels, respectively; Supplementary Figures 3A and B) attenuated the dexamethasone induction of these mRNAs (Figure 4) with little or no effect of scrambled siRNA on dexamethasone-induced transcriptional responses (Supplementary Figure 3C). These experiments confirm GR as a crucial mediator of the transcriptional responses to glucocorticoids in foetal cardiomyocytes. To examine direct regulation by GR, cycloheximide was administered prior to dexamethasone, to block new protein synthesis. Glucocorticoid-induction of *Tsc22d3* and *Ppargc1a* mRNA was maintained (and increased in the case of *Ppargc1a*) with cycloheximide, suggesting these genes are direct targets of GR in cardiomyocytes (Figures 4a and d). In contrast, glucocorticoid induction of *Myh6* and *Nppa* mRNAs was abolished by cycloheximide demonstrating this regulation depends on new protein synthesis (Figures 4b and c).

RNAseq analysis reveals potential primary glucocorticoid targets in cardiomyocytes. The above experiments suggest GR initiates a transcriptional cascade in foetal cardiomyocytes, in which master regulators (such as *Tsc22d3* and *Ppargc1a*) are induced to establish the maturational programme. To explore the extent of the glucocorticoid-induced programme and to identify potential
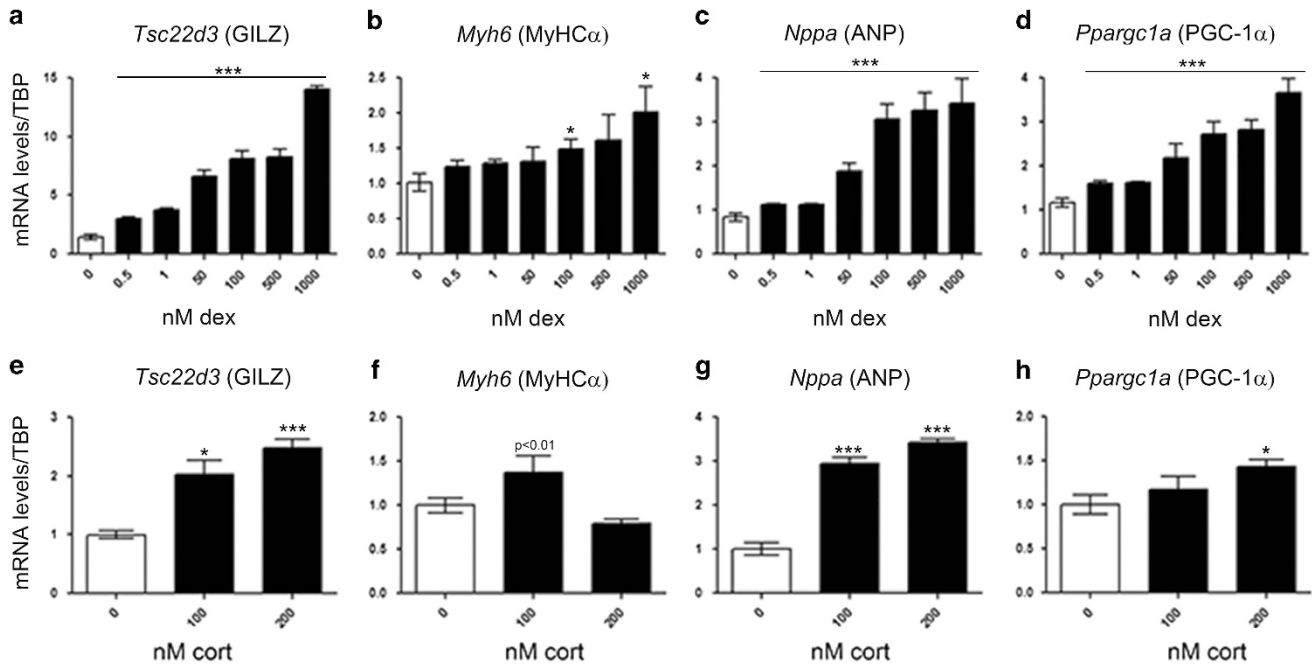
Figure 3 Glucocorticoid treatment of primary foetal cardiomyocytes dose-dependently replicates *in vivo* regulation of cardiac gene expression by glucocorticoid action. Treatment of primary foetal C57BL/6 cardiomyocytes for 24 h with dexamethasone (dex) (**a–d**) or corticosterone (cort) (**e–h**) dose-dependently increased levels of mRNA encoding glucocorticoid-induced leucine zipper (GILZ), MyHCα, atrial natriuretic peptide (ANP) and PGC-1α. Data were analysed by one-way ANOVA with Bonferroni *post-hoc* test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $n = 5$ replicates over two independent experiments

master regulators and primary target genes of GR in foetal heart, next generation RNA sequencing (RNAseq) was performed on primary foetal cardiomyocytes following treatment with 100 nM dexamethasone for 2 h in the presence of cycloheximide. Controls were treated with cycloheximide alone. A total of 613 transcripts covering 564 genes were differentially expressed (adjusted $P < 0.05$) in glucocorticoid-treated cardiomyocytes, with 469 transcripts increased and 144 decreased. As expected, the induced genes included *Ppargc1a* and *Tsc22d3* (Table 1). Gene set enrichment analysis with gene ontology (GO) terms revealed sequence-specific DNA binding proteins with transcription factor activity, and sequence-specific DNA binding proteins to be the two groups of most over-represented terms affecting molecular function, representing ~9% of differentially expressed genes (Table 1; see Supplementary Table 1 for the full list). These include regulators of key pathways including development and growth (*Notch1, Myc, Wt1, Hes1*), circadian rhythm (*Per1, Nr1d1*), metabolism (*Cebpb, Ppara*), and hypoxia (*Hif3a, Epas1*), several of which are known GR targets in other cell types (e.g., *Hes1, Notch1, Per1, Ppara*[17–20]). *Nr3c1*, encoding GR itself, was repressed, though *Nr3c2* (encoding MR) was unaffected by dexamethasone. *Nr0b2*, encoding small heterodimer partner (SHP), a repressor of nuclear receptor function and regulator of both PGC-1 and GR activity,[21] was also included in this group. Several induced genes encode factors implicated in early cardiac development, including *Klf2* and *Notch1.* Our data suggest they may also be induced by glucocorticoid to play a later role in cardiac maturation. Although *Klf2* mRNA was unaltered in hearts of E17.5 GR^{−/−} foetuses,[5] *Klf2* might be transiently induced in the wild-type heart as corticosterone levels

increase at E15.5, with subsequent negative feedback returning levels to basal by E17.5. Such temporary increases in key developmental or transcriptional regulators can lead to permanent changes in cellular transcriptional programmes, setting the cell on the path to terminal differentiation.[22] It is also possible these effects on expression of developmental regulators relates to the *in vitro* conditions used here.

Other transcriptional regulators, which themselves are not DNA binding proteins, were differentially expressed. These include reciprocal regulation of *Ppargc1a* (increased twofold) and *Ppargc1b* (decreased to almost the same extent). Current evidence suggests PGC-1β plays a redundant role in foetal heart maturation.[23] However, PGC-1β expression declines as PGC-1α expression increases during cardiomyocyte differentiation of human ES cells (hESC),[24] consistent with our data. Other repressed genes include *Bmp4, Hey1,* and *Efna1,* encoding key regulators of early cardiac differentiation,[25–29] in accordance with the maturational effects of glucocorticoids on foetal cardiomyocytes.

Other differentially expressed genes include cell surface growth factor or neurotransmitter receptors as well as several members of the voltage-gated potassium channel family (e.g., *Kcna5, Kcnk1, Kcne4, Kcnk5*) and subunits of the ENaC epithelial sodium channel. Members of the voltage-gated potassium channel family have previously been identified as glucocorticoid-regulated genes,[30–32] supporting their identification here as primary targets of GR in foetal cardiomyocytes. Several members of the Slc family of solute transporters were differentially expressed suggesting altered transport of small molecules across membranes. These include four members of the *Slc25* family of mitochondrial carriers, *Slc25a25, Slc25a33, Slc25a18,* and *Slc25a30*, two of which transport
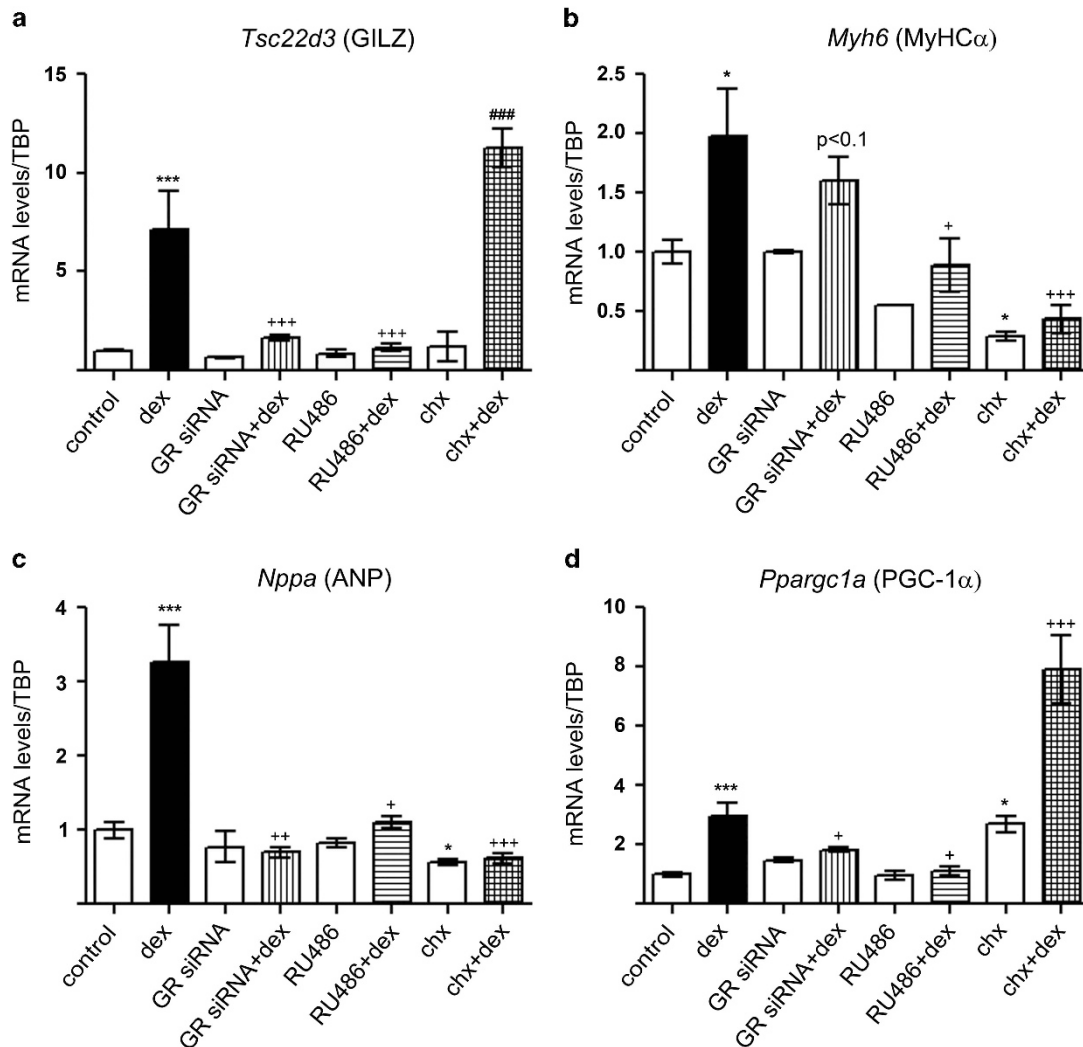
Figure 4 Glucocorticoids act *via* GR to exert transcriptional effects on primary-mouse-foetal cardiomyocytes. GR siRNA or pretreatment with RU486 (10 μM, 30 min) blocked or attenuated the dexamethasone (1 μM, 24 h) induction of mRNAs encoding GILZ (**a**), ANP (**b**), MyHCα (**c**) and PGC-1α (**d**). Cycloheximide (CHX, 10 μM, 30 min) pretreatment blocked the glucocorticoid induction of MyHCα and ANP, but not GILZ or PGC-1α. Data were analysed by two-way ANOVA with Bonferroni multiple comparisons *post-hoc* test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* untreated; +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ *versus* dex; $n = 4$ replicates. Statistical significance *versus* dex is only indicated for dexamethasone-treated groups

nucleotides. Thus, glucocorticoids directly affect some aspects of mitochondrial function whereas other may be secondary to induction of "master regulators" such as PGC-1α.

PGC-1α mediates the effects of glucocorticoids on myofibrillar structure and oxygen consumption of cardiomyocytes. We validated PGC-1α as an early target of glucocorticoid action in foetal cardiomyocytes by confirming dexamethasone induction of *Ppargc1a* mRNA within 2 h as well as 8 h of dexamethasone addition. Although steady-state *Ppargc1a* mRNA levels increased following addition of cycloheximide (suggesting rapid turnover of *Ppargc1a* mRNA), dexamethasone further increased *Ppargc1a* mRNA levels, above those with cycloheximide alone (Supplementary Figures 3D and E), consistent with *Ppargc1a* being a direct target of GR. *In vivo*, *Ppargc1a* mRNA levels are reduced in hearts of GR^{−/−} mice[5] and like GR^{−/−} foetuses, mice lacking PGC-1α and PGC-1α show profound defects in foetal cardiac

maturation with functionally immature hearts and persistence of a foetal-gene expression pattern, including reduced expression of *Atp2a2* and *Myh6*.[23] *Ppargc1a* is normally induced at E15.5[23] co-incident with the rise in cardiac corticosterone levels, which are negligible at E14.5.[5] To test whether glucocorticoids can induce *Ppargc1a in vivo*, dexamethasone was injected into pregnant dams at E14.5. Within 6 h, steady-state levels of *Ppargc1a* mRNA were increased in foetal hearts, as were levels of *Fkbp5* mRNA, a well-known GR target (Figures 5a and b), confirming glucocorticoid regulation *in vivo*. To determine whether PGC-1α induction is critical for glucocorticoids to elicit foetal cardiomyocyte maturation, PGC-1α siRNA was used to reduce *Ppargc1a* mRNA levels $\sim 60\%$, abolishing the normal increase in *Ppargc1a* mRNA levels following glucocorticoid treatment (Supplementary Figure 4A). This reduction of *Ppargc1a* mRNA in glucocorticoid-treated cardiomyocytes, to reach the same level as in untreated cells, markedly

Table 1 Differentially expressed genes were subject to gene set enrichment analysis with gene ontology terms affecting molecular function

P-value	Expected count	Count	Size	Genes (fold-change)
GO:0003700: sequence-specific DNA binding transcription factor activity				
1.392×10^{-6}	14.523	35	393	Klf5 (3.07), Runx1t1 (2.01), Cebpb (3.13), Arid3a (−1.67), Elf3 (−4.92), Epas1 (1.51), Erf (3.45), Tsc22d3 (2.11), Nr3c1 (−1.47), Gsc (18.48), Hand2 (1.58), Hes1 (−3.73), Hey1 (−3.38), Hlx (3.43), Id3 (−2.01), Irf1 (−1.61), Klf2 (1.72), Mafb (−3.21), Lhx6 (7.40), Mycl1 (6.53), Aff1 (2.15), Mnt (−1.85), Myc (2.09), Nfe2l3 (−1.68), Mycn (−1.47), Notch1 (2.07), Pitx3 (38.36), Ppara (2.93), Prox1 (−1.72), Six1 (2.56), Sox12 (−1.55), Sox4 (1.97), Bhlhe40 (−1.99), Tfdp2 (2.88), Csrnp1 (2.22), Hlf (2.08), Nr1d1 (−1.62), Rarb (−1.62), Vdr (4.02), Wt1 (2.10), Tgif2 (−1.68), Grhl3 (1.81), Zbtb16 (61.17), Nr0b2 (1.62), Hif3a (7.36), Heyl (−2.01), Foxo3 (2.68), Elf4 (−1.81), Klf15 (3.07), Tcfap4 (−1.70), Glis2 (−1.91)
GO:0043565: sequence-specific DNA binding				
2.48×10^{-5}	17.238	36	458	Cebpb (3.13), Elf3 (−4.92), Epas1 (1.51), Erf (3.45), Nr3c1 (−1.47), Gsc (18.48), Hand2 (1.58), Hes1 (−3.73), Hey1 (−3.38), Hlx (3.43), Irf1 (−1.61), Klf2 (1.72), Mafb (−3.21), Lhx6 (7.40), Myc (2.09), Nfe2l3 (−1.68), Notch1 (2.07), Per1 (6.08), Pitx3 (38.36), Ppara (2.93), Prox1 (−1.72), Scx (−1.61), Six1 (2.56), Sox12 (−1.55), Sox4 (1.97), Hlf (2.08), Nr1d1 (−1.62), Rarb (−1.62), Vdr (4.02), Wt1 (2.10), Tgif2 (−1.68), Heyl (−2.01), Foxo3 (2.68), Elf4 (−1.81), Ctcfl (2.13), Tcfap4 (−1.70)
GO:0070888: E-box binding				
4.932×10^{-5}	0.715	6	19	Hand2 (1.58), Myc (2.09), Per1 (6.08), Scx (−1.61), Bhlhe40 (−1.99), Tcfap4 (−1.70)
GO:0000981: sequence-specific DNA binding RNA polymerase II transcription factor activity				
0.0007	6.436	16	171	Cebpb (3.13), Hand2 (1.58), Hes1 (−3.73), Ppara (2.93), Prox1 (−1.72), Sox12 (−1.55), Sox4 (1.97), Bhlhe40 (−1.99), Rarb (−1.62), Vdr (4.02), Wt1 (2.10), Grhl3 (1.81), Heyl (−2.01), Foxo3 (2.68), Tcfap4 (−1.70), Glis2 (−1.91)
GO:0000975: regulatory region DNA binding				
0.0021	9.823	20	261	Nr3c1 (−1.47), Hand2 (1.58), Hes1 (−3.73), Hlx (3.43), Irf1 (−1.61), Myc (2.09), Per1 (6.08), Prox1 (−1.72), Scx (−1.61), Six1 (2.56), Sox12 (−1.55), Sox4 (1.97), Bhlhe40 (−1.99), Nr1d1 (−1.62), Wt1 (2.10), Heyl (−2.01), Ctcfl (2.13), Tbl1xr1 (1.83), Tcfap4 (−1.70), Glis2 (−1.91)
GO:0001077: RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor				
0.0028	1.9195	7	51	Cebpb (3.13), Hand2 (1.58), Sox12 (−1.55), Sox4 (1.97), Wt1 (2.10), Heyl (−2.01), Glis2 (−1.91)
GO:0000988: protein binding transcription factor activity				
0.0074	11.742	21	312	Elf3 (4.92), Lpin1 (1.66), Nr3c1 (−1.47), Hand2 (1.58), Hey1 (−3.38), Jub (−2.14), Ppargc1b (−1.85), Per1 (6.08), Per2 (1.95), Ppargc1a (1.97), Prox1 (−1.72), Sox12 (−1.55), Sox4 (1.97), Bhlhe40 (−1.99), Nr0b2 (1.62), Irf2bp2 (1.88), Heyl (−2.01), Cited4 (−1.58), Sertad2 (−1.62), Med26 (2.46), Tbl1xr1 (1.83)
GO:0008134: transcription factor binding				
0.0153	13.361	22	355	Cebpb (3.13), Epas1 (1.51), Lpin1 (1.66), Tsc22d3 (2.11), Nr3c1 (−1.47), Hand2 (1.58), Hes1 (−3.73), Id3 (−2.01), Id4 (2.21), Mafb (−3.21), Myc (2.09), Nab2 (−1.97), Nfkbia (3.04), Ppara (2.93), Ppargc1a (1.97), Scx (−1.61), Bhlhe40 (−1.99), Tfdp2 (2.88), Vdr (4.02), Nr0b2 (1.62), Foxo3 (2.68), Tob2 (5.03)
GO:0003712: transcription cofactor activity				
0.0427	10.764	17	286	Elf3 (4.92), Lpin1 (1.66), Nr3c1 (−1.47), Hand2 (1.58), Jub (−2.14), Ppargc1b (−1.85), Ppargc1a (1.97), Prox1 (−1.72), Sox12 (−1.55), Sox4 (1.97), Nr0b2 (1.62), Irf2bp2 (1.88), Heyl (−2.01), Cited4 (−1.58), Sertad2 (−1.62), Med26 (2.46), Tbl1xr1 (1.83)

GO terms are indicated, with expected and actual gene counts and *P*-value. Differentially expressed genes are listed with fold-change (dex/control; repression shown as a negative fold-change) indicated in brackets

attenuated the effect of corticosterone on myofibrillar structure, sarcomere length and Z-disc width (Figure 6). In contrast, PGC-1α knock-down had no significant effect on glucocorticoid induction of *Tsc22d3*, *Nppa*, or *Myh6* (Supplementary Figure 3C), nor, importantly, did it affect *Nr3c1* (GR) mRNA or protein levels (Supplementary Figure 3B).

The GR-mediated induction of PGC-1α increases oxygen consumption rate in primary foetal cardiomyocytes.

The known role of PGC-1α in mitochondrial gene expression[33] suggested that glucocorticoids promote mitochondrial function and/or capacity in foetal cardiomyocytes. To test this, the oxygen consumption rate (OCR) was measured following 100 nM corticosterone treatment for 24 h. OCR was higher in corticosterone-treated cells, an effect attenuated by RU486 (Figure 7a) indicating GR activation increases cellular respiration. Knock-down of PGC-1α eliminated the effect of corticosterone on OCR but had no effect in its absence (Figure 7b), demonstrating that the glucocorticoid-mediated promotion of mitochondrial function as well as cardiomyocyte structure depends upon PGC-1α induction. Corticosterone also enhanced the mitochondrial capacity of foetal cardiomyocytes as shown by a greater increase in OCR following addition of 2,4-dinitrophenol to uncouple mitochondrial respiration from ATP synthesis (Supplementary Figure 5).
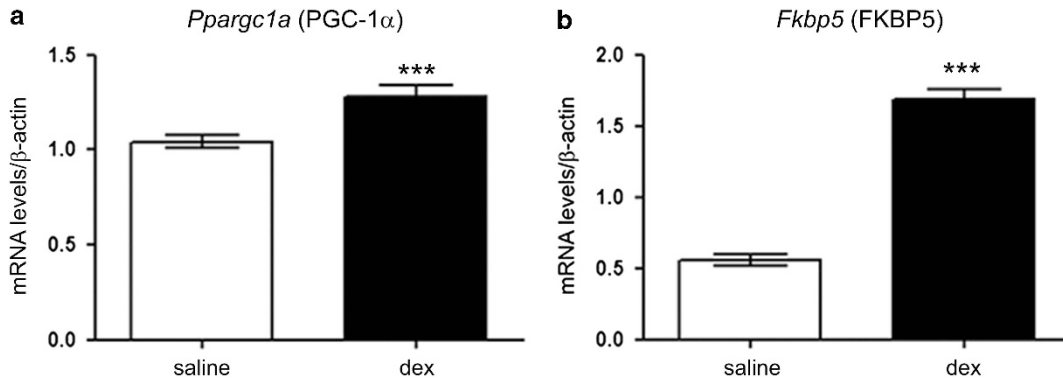
Figure 5 Dexamethasone increases steady-state levels of Ppargc1a (PGC-1α) and Fkbp5 mRNA after 6 h in E14.5 foetal heart. Dexamethasone (500 μg/kg body weight) was administered to pregnant mothers by peritoneal injection at E14.6. Foetal tissues were collected after 6 h. Data were analysed by Student's t-test; ***$P < 0.001$; $n = 22$ foetuses (from six pregnant dams per group)
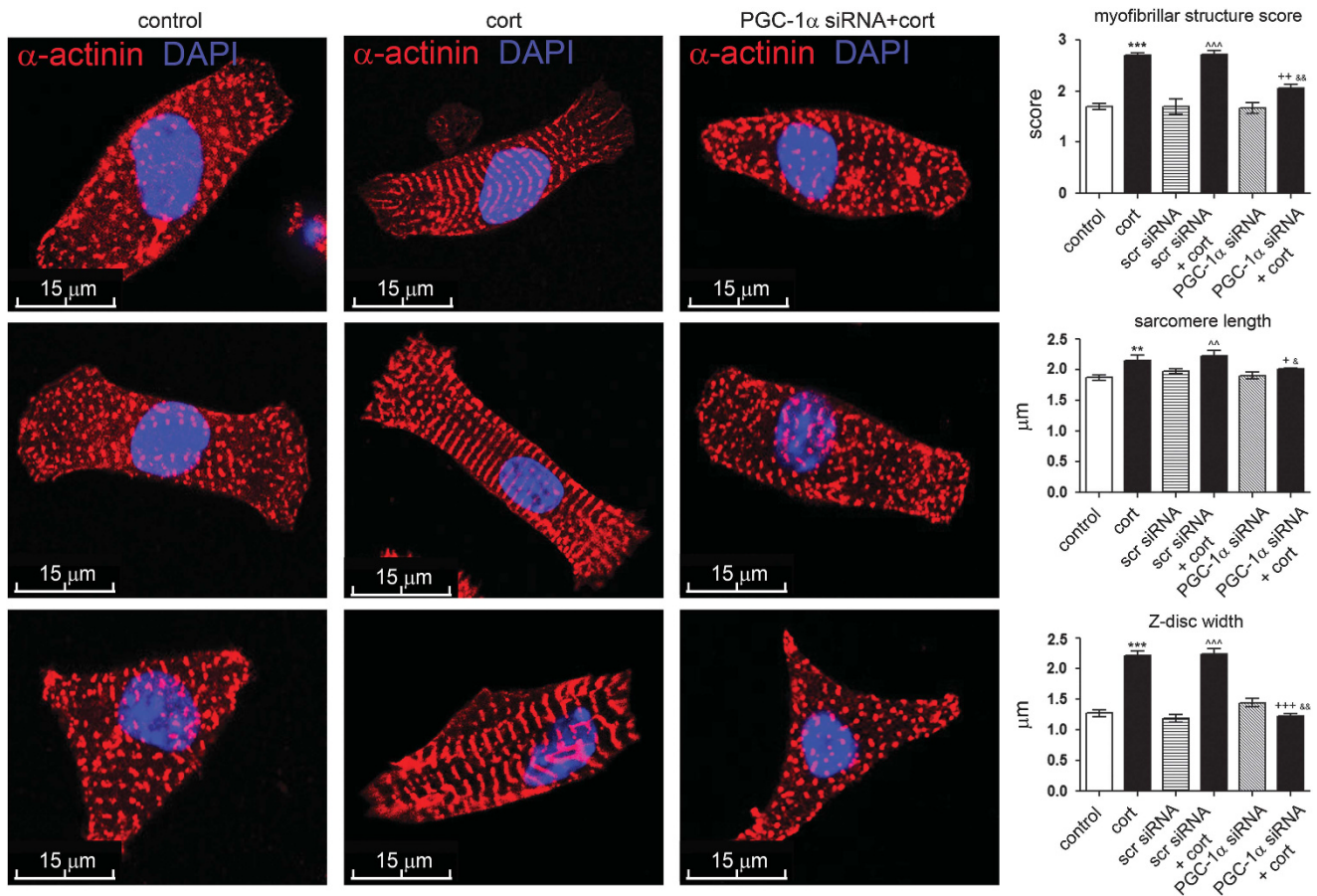

Figure 6 Corticosterone-induced maturation of myofibril structure is abrogated by PGC-1α knock-down. Cells were untreated (control; left panels) or treated with 100 nM corticosterone for 24 h (cort; centre panels). To block PGC-1α-mediated effects, cells were transfected with PGC-1α siRNA 40 h prior to glucocorticoid treatment (right panels). Cells were fixed and stained for α-actinin (red). DAPI (blue) was used as a nuclear counterstain. Images representative of $n = 25$–120 cells. Images were also scored for myofibrillar structure (as described in Materials and Methods). PGC-1α knock-down abrogated corticosterone induced increases in myofibrillar structure score (top graph), sarcomere length (middle graph) and Z-disc width (bottom graph). Data were analysed by one-way ANOVA with Bonferroni's *post-hoc* test; $^{+/\&}P < 0.05$, **/++/^^/&&$P < 0.01$, ***/+++/^^^$P < 0.001$; *versus* untreated control, $^{+}$*versus* dex, $^{\wedge}$*versus* scambled PGC-1α siRNA, and *versus* PGC-1α siRNA; $n = 25$–120 replicates over two independent experiments
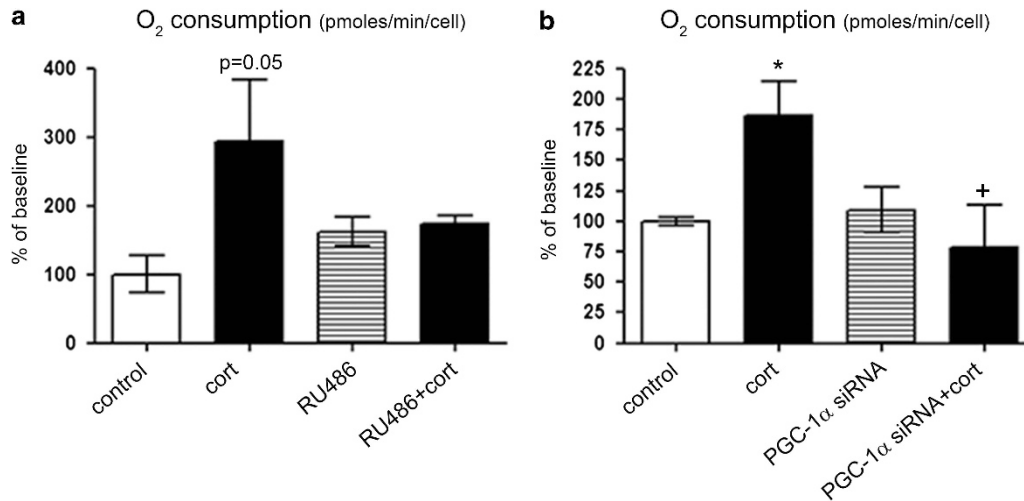
Figure 7 Glucocorticoids act *via* GR and PGC-1α to increase mitochondrial respiration in primary foetal cardiomyocytes. Real-time oxygen consumption rate (OCR) was measured in untreated (control) foetal cardiomyocytes or following treatment with 100 nM corticosterone for 24 h (cort). Cells were pre-treated with RU486 (1 μM, 30 min) or transfected with PGC-1α siRNA (40 h). Data were analysed by one-way ANOVA with Bonferroni's *post-hoc* test; *$P < 0.05$ *versus* control, +$P < 0.05$ *versus* cort

Discussion

At a physiologically relevant concentration, corticosterone improved the contraction-relaxation properties of primary foetal cardiomyocytes and elicited a striking maturation of myofibrillar content and organisation. This mirrors the *in vivo* situation where the absence of GR signalling in foetal cardiomyocytes impairs contraction and results in short, disorganised myofibrils with poorly defined Z-discs,[5] suggesting similar mechanisms for glucocorticoid maturation of cardiomyocyte structure and function *in vivo* as *in vitro*.

The glucocorticoid-mediated improvement in contraction-relaxation properties is likely underpinned by improved calcium handling as well as structural changes in myofibrils. As observed *in vivo*,[5] glucocorticoid action increased expression of genes encoding the calcium handling proteins SERCA2a and RYR2 as well as contractile proteins (MyHCα) in primary foetal cardiomyocytes. This glucocorticoid induction of mRNAs encoding proteins involved in calcium-induced calcium-release accords with previous findings that glucocorticoids increase cardiac L- and T-type calcium current amplitude and channel density in rat neonatal cardiomyocytes.[34–37] Moreover, cardiomyocytes isolated from E18.5 GR$^{-/-}$ foetuses have lower peak calcium channel amplitude,[38] further supporting a role for glucocorticoid action in regulating sarcolemmal calcium flux. Apart from genes involved in cardiomyocyte depolarisation and initiation of contraction, our data suggest glucocorticoids also promote the later stages of cardiac excitation-contraction coupling, as dexamethasone increased mRNA encoding NCX and several voltage-gated potassium channels, vital for cardiomyocyte repolarisation. However, whilst the RNAseq implicated genes encoding potassium channels as primary GR targets, genes encoding calcium handling proteins were not differentially expressed, and hence their induction by glucocorticoids in foetal cardiomyocytes is most likely a secondary effect of GR signalling.

Glucocorticoid treatment dramatically improved myofibril appearance in foetal cardiomyocytes. The redistribution of

α-actinin, from the diffuse punctate staining pattern of untreated foetal cardiomyocytes to the well-defined Z-discs in corticosterone-treated cells probably reflects the fusion of Z-bodies of immature pre-myofibrils to form the Z-discs in mature myofibrils.[39] In a previous electron microscopy study, contractile filaments were confined to the periphery of rat foetal (E20) myocytes and lacked sarcomeres whereas neonatal rat myocytes contained sarcomeres,[8] consistent with more mature myofibril structure in neonatal cardiomyocytes compared to foetal. This structural aspect of glucocorticoid-promoted cardiomyocyte maturation depends on glucocorticoid induction of *Ppargc1a* though other aspects of cardiomyocyte maturation also seen *in vivo* (*Myh6* and *Nppa* induction) are not and are presumably mediated by other GR-induced transcription regulators. In a recent report, knockdown of PGC-1α or uncoupling of mitochondrial activity by 2,4-dinitrophenol in hESC cardiomyocytes diminished the α-actinin banding pattern of contractile filaments,[24] an effect strikingly similar to that we observed following PGC-1α knockdown in glucocorticoid-treated foetal cardiomyocytes. Given that PGC-1α knock-down also reduced mitochondrial volume and activity in hESC cardiomyocytes,[24] this strongly suggests that a PGC-1α-driven increase in mitochondrial capacity promotes contractile function through improved sarcomeric integrity, as cardiomyocytes mature. Indeed, glucocorticoid treatment increased basal respiration in foetal cardiomyocytes in a PGC-1α-dependent manner, consistent with the PGC-1α-driven effects on both myofibrillar structure and mitochondrial capacity in hESC cardiomyocytes[24] as well as data from rat neonatal cardiomyocytes showing that dexamethasone increases both intracellular ATP levels and capacity to synthesise ATP.[40] PGC1-α also promotes production of reactive oxygen species (ROS) in hESC-derived cardiomyocytes.[24] Interestingly, FOXO3, important for resistance to oxidative stress in cardiomyocytes,[41] was increased in our RNAseq, suggesting glucocorticoid action concurrently induces the processes that generate ROS as well as mechanisms to alleviate the potential damage they may

cause. PGC-1α is already expressed in mouse foetal heart at E15.5,[23] suggesting it may act as a GR coactivator[42,43] and/or acts downstream of GR to enhance metabolic maturation of foetal cardiomyocytes. Our data support PGC-1α as downstream and a direct target of GR but do not eliminate a role as a coactivator of GR. Indeed, a recent study in mouse islets showed that PGC-1α is both a target and a co-regulator of GR, with downstream effects dependent on the presence of both at regulated promoters.[44] A similar co-dependence in foetal cardiomyocytes might ensure that although expressed, PGC-1α does not exert its full biological effects until corticosterone levels rise and GR is activated. How GR induces *Ppargc1a* is currently unclear. We were unable to show glucocorticoid regulation of luciferase reporter constructs containing ~4 kb of the *Ppargc1a* promoter, despite it being glucocorticoid inducible in mouse BWTG3 hepatoma cells (unpublished data), thus the glucocorticoid responsive element must lie outside of this region.

The postnatal heart heavily relies on oxidative catabolism of fatty acids to produce energy.[45] Our data support the view that glucocorticoid action in late gestation drives this maturation of cardiac energy metabolism. In addition to PGC-1α induction and independently of new protein synthesis, dexamethasone increased expression of master regulators of lipid/fatty acid oxidation in foetal cardiomyocytes: PPARα, Klf15, and lipin 1. PPARα co-operates with PGC-1α to induce genes required for fatty acid uptake and oxidation in the myocardium[46] Lipin-1, an amplifier of PGC-1α/PPARα mediated control of lipid metabolism that promotes fatty acid oxidation and mitochondrial oxidative phosphorylation in the liver[47] may play a similar role in heart, where it is both a transcriptional target of, and coactivator of, PGC-1α.[48] Klf15, a known GR target, regulates amino acid, lipid, and glucose metabolism[49–51] and acts with GR to control feed-forward gene-regulatory circuits in lung.[52] Klf15 is also a direct regulator of myocardial lipid flux, induced in the mouse heart in the perinatal period.[53] Thus glucocorticoid action induces a set of master transcriptional regulators which themselves may act in concert with GR in feed-forward circuits to increase cardiomyocyte ATP-generating capacity. As well as master regulators of fatty acid oxidation, dexamethasone markedly induced *Plin4*, encoding perilipin 4, a lipid droplet coating protein. *Plin4* disruption in mice reduces cardiac-lipid content,[54] suggesting glucocorticoids promote storage of energy substrate in cardiomyocytes as well as increasing capacity to utilise it. It is interesting in this respect that several Slc transporters were glucocorticoid-induced in the presence of cycloheximide, suggesting increased substrate flux into and around the cell as part of a co-ordinated glucocorticoid-mediated increase in energy-generating capacity. A substantial minority of differentially expressed genes were decreased following dexamethasone treatment in the presence of cycloheximide. How this reduction in steady-state mRNA levels occurs within 2 h is unclear. However, the repressed genes include *Hes1*, a highly conserved transcriptional repressor required for organogenesis[55] and a master regulator of GR-dependent gene expression.[56] In liver, GR-dependent antagonism of NFκB at the *Hes1* locus, rapidly silences HES1, increasing glucocorticoid sensitivity and altering lipid and carbohydrate metabolism.[56] Decreased

Hes1 expression in foetal cardiomyocytes may similarly enhance glucocorticoid action, aiding metabolic maturation.

A number of the genes that we identified as glucocorticoid regulated in foetal cardiomyocytes are glucocorticoid regulated in other muscle-related cell types, including rat neonatal cardiomyocytes, embryonic rat H9C2 myoblasts and mouse C2C12 myotubes.[30–32] However, despite similarities between glucocorticoid-regulated genes in this and previous studies, none show the same pattern of glucocorticoid-regulated gene regulation, with the cell lines showing many differences to foetal cardiomyocytes. Differences to neonatal rat cardiomyocytes may reflect their prior exposure to glucocorticoid, possibly altering subsequent responses.[9] Remarkably, of the 173 glucocorticoid-induced genes identified as direct targets of GR in C2C12 cells, only a third of the induced genes and none of the repressed genes showed similar regulation in foetal mouse cardiomyocytes; three even showed the opposite regulation. Although some of this variation may relate to experimental conditions, the data are consistent with previous demonstrations of the highly cell-specific repertoire of GR-regulated genes,[10,57,58] and illustrate the importance of establishing the glucocorticoid-regulated transcriptome within the relevant primary cell.

These data have wider implications. Despite the routine use of glucocorticoids in preterm babies, little is known of how effects on heart contribute to improved survival of neonates. Our experiments have identified early molecular events triggered by glucocorticoid action in cardiomyocytes during late foetal development and highlight mechanisms likely to be critical for maturation and thus resilience of the foetal heart, in the perinatal period.

Materials and Methods

Animals; *in vivo* dexamethasone administration. All animal experimentation was carried out in strict accord with accepted standards of humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986 after prior approval by the University of Edinburgh Animal Ethical Review Committee.

Female C57BL/6 mice were maintained under conditions of controlled lighting and temperature. Time-mated females were injected intra-peritoneally with 500 μg/kg dexamethasone or vehicle (saline) at E14.5 then culled 6 h post-injection. Foetal hearts were collected and stored at − 80 °C prior to RNA analysis.

Cardiomyocyte cultures. Primary mouse (*Mus musculus*) foetal cardiomyocytes were isolated from the ventricles of E15.5–E17.5 C57BL/6 foetal hearts and cultured as described,[59] except that 0.3 mg/ml collagenase II (Worthington, Lakewood, NJ, USA) was added to the enzyme buffer and cardiomyocytes were cultured on gelatin-covered plates. Isolated cells were ≥ 98% troponin T-positive cells (Supplementary Figure 1A) and when cultured, cells proliferated and beat spontaneously and synchronously after ~2 days. Cells were treated with dexamethasone or corticosterone (as stated in the figure legends), with 30 min pretreatment with 10 μM cycloheximide or RU38486 (mifepristone) where appropriate (all purchased from Sigma; Poole, UK). Control cells were treated with vehicle only (96% ethanol). For siRNA knock-down, 40 h prior to dexamethasone administration cells were transfected with GR or PGC-1α siRNA (catalogue number s67065 and s72015, respectively; Ambion, Austin, TX, USA) using Lipofectamine 2000 (Life Technologies, Paisley, UK) according to the manufacturer's instructions.

Soft-video edge detection. Mechanical properties of spontaneously contracting cardiomyocytes were measured using an optical video edge-detection system (Ionwizard, Ionoptix, Milton, MA, USA) with a heated stage (37 °C). Analysis of magnitude and timecourse was performed with Origin6.1 (Originlabs Corporation, Northampton, MA, USA).

Immunohistochemistry and immunofluorescence. Cells grown on glass coverslips were fixed in 4% paraformaldehyde and stained according to standard protocols with anti-α-actinin antibody (Abcam, Cambridge, UK) or anti-α-smooth muscle actin (α-SMA) antibody (Sigma) and troponin T (Abcam). Fluorophore-conjugated secondary antibodies (Alexa Fluor, Life Technologies) were used to detect immunostaining. Scoring of myofibrillar structure was carried out on images by two or three people not involved in the experiment, who were blind to treatment group, according to the following scores: 1 = randomly scattered punctuate or fuzzy appearance of Z-structures; 2 = clearly visible sarcomeres (striation, but myofibrils not well organised with indistinct Z-structures; and 3 = clearly visible, distinct striation and wide discrete Z-discs, high degree of myofibril alignment.

Electron microscopy. Cells grown on plastic coverslips were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, then dehydrated and embedded in araldite resin. Ultrathin (60 nm) sections were cut and stained in uranyl acetate and lead citrate then viewed in a Phillips CM120 Transmission electron microscope (FEI UK, Cambridge, England).

Quantitative (q)PCR. RNA was extracted using a PureLinkTM RNA Mini kit (Life Technologies) according to the manufacturer's instructions and reverse transcribed using a Superscript First Strand Synthesis kit (Life Technologies). Quantitative (q)PCR was carried out using a LightCycler 480 (Roche Diagnostics, Burgess Hill, UK) with a commercial master mix (LC 480 Probes Master) using the Universal Probe Library system (Roche Diagnostics; Table 2). Data were analyzed using the second derivative maximum method. The ratio of levels of the transcript of interest to levels of TATA Binding Protein (TBP) or β-actin mRNA (as internal control; see figure legends) was determined for each sample.

Western blotting. GR levels were measured by western blot using 1 : 400 dilution rabbit anti-mouse GR primary antibody (M-20, Santa Cruz Biotechnology, Dallas, TX, USA). Protein levels were measured relative to β-tubulin levels using an Odyssey infra-red imaging system (Licor Biosciences, Lincoln, NE, USA).

RNAseq. RNAseq was performed by Edinburgh Genomics (University of Edinburgh) on RNA extracted from primary foetal cardiomyocytes treated with 100 nM dexamethasone for 2 h in the presence of 10 μM cycloheximide. Control cells were treated with cycloheximide alone. TruSeq (version 2; Illumina, San Diego, CA, USA) libraries were prepared and sequenced using an Illumina HiSeq 2000 platform (Illumina UK, Little Chesterford, Essex, UK), generating 50 base single-end sequences, yielding 30–50 M reads per sample. Reads were mapped using GSNAP and assigned to exon features with the htseq-count utility of HTSeq[60–62] (version 0.5.3) in 'union' mode, specifying a non-stranded library, a minimum quality of 20 and mouse genome version NCBI m37 (Ensembl 67) as reference annotation. Counts were then normalised by effective library size, and differential expression (count) assessed, with the use of the DESeq package[60] (v. 1.9.7) from Bioconductor.[61] Normalised counts and differential expression measures were placed in a database with in-house tools for data mining. Data were analysed based on fold-change and statistical significance. Enrichment of GO terms in differentially expressed gene sets was assessed with the hypergeometric statistic as implemented in the GOstats package[62] (v 2.24.0) of Bioconductor.

Cellular bioenergetics analysis. OCR values were measured in real time in primary foetal cardiomyocytes using a Seahorse XF-24 bioanalyser (Seahorse Bioscience, North Billerica, MA, USA). Cells were seeded at a density of ~ 75 000 per well in a XF-24 microplate 24 h prior to analysis and treated with corticosterone and/or RU38486 or transfected with PGC-1α siRNA as described above. One hour prior to analysis media was changed to unbuffered DMEM and cells were incubated at 37 °C in 0% CO_2. Analysis was performed according to the manufacturer's instructions using an XF assay kit (Seahorse BioScience). Three OCR measurements (performed every 6 min) were recorded. To measure mitochondrial reserve, following measurement of OCR, 100 μM 2,4-dinitrophenol was injected and then two subsequent measurements were made. Following the assay, cells were trypsinized and counted, and the OCR values corrected for the cell number.

Statistics. All numerical data are presented as mean ± S.E.M. Statistical evaluation was carried out with Prism (GraphPad) and used Students t-test, one-way ANOVA with Bonferroni's *post-hoc* test or two-way ANOVA with Bonferroni's multiple comparisons *post-hoc* test, or area under the curve analysis, as appropriate.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

EAR-Z, CJK, DGH, MCH, KEC (conception), EAR-Z, KEC (design), EAR-Z, M-AC, RVR, GJG, KG (execution), EAR-Z, JRM, GJG, DRD, CJK, GLS, MCH, KEC (interpretation), EAR-Z, KEC (manuscript preparation).

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