Dexamethasone and sex regulate placental glucocorticoid receptor isoforms in mice

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Abstract

Maternal dexamethasone exposure in the mouse impairs placental development and programs adult disease in a sexually dimorphic manner. Glucocorticoids bind to different glucocorticoid receptor (GR) isoforms to regulate gene transcription and cellular signaling. We hypothesized that sexually dimorphic placental responses to glucocorticoids are due to differences in GR isoforms present in the placenta. Pregnant C57Bl6 mice were exposed to saline or dexamethasone from E12.5 until E14.5 (1 µg/kg/h) before the collection of placentae. Cytoplasmic and nuclear protein fractions were extracted from placentae of male and female fetuses for Western blot analysis of GR isoforms. Eight known isoforms of the GR were detected in the mouse placenta including the translational isoforms GR α -A, B, C and D1–3 and the splice variants GRA and GRP. The expression of GRA, GRP and each of the GR α isoforms were altered by dexamethasone in relation to fetal sex and cellular location. Placentae of female fetuses had higher GR α -A and GRP expression in the cytoplasm than males, and GR α -C was more highly expressed in the nucleus of females than that in males. Dexamethasone significantly increased the cytoplasmic expression of GR α -A, but reduced the expression of GR α -C in placentae of males. Dexamethasone increased the expression of the GR α -C-regulated genes *Sgk1* and *Bcl2l11*, particularly in females. The cleaved caspase-3 staining in placental sections indicated GR α -C may mediate sex differences in dexamethasone-induced apoptosis. These findings may underlie the sex-specific placental adaptations that regulate different growth profiles in males and females and different risks for programmed disease outcomes in offspring.

Key Words

- ▶ apoptosis
- ▶ oxidative stress
- ▶ fetal programming
- ▶ stress hormone
- ▶ junctional zone

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Introduction

Maternal glucocorticoid exposure programs offspring disease in a sexually dimorphic manner (O'Regan *et al.* 2004, Singh *et al.* 2012). This is in part due to sexually dimorphic placental responses to glucocorticoids during

pregnancy, which mediate different fetal growth strategies (Audette *et al.* 2010, Clifton 2010, Eriksson *et al.* 2010, O'Connell *et al.* 2011, Vaughan *et al.* 2012). We have reported that maternal dexamethasone exposure in the

mouse reduces weight in both male and female fetuses but reduces placental weight in female fetuses only (Cuffe *et al.* 2011). The reduction in placental weight of females was associated with a reduction in the size of the junctional zone but an increase in the glucocorticoid metabolizing enzyme hydroxysteroid 11- β -dehydrogenase isozyme 2 (hsd11b2). These female adaptations likely prevent the cardiovascular and renal dysfunction that develop only in male offspring (O'Sullivan *et al.* 2013).

Normal regulation of glucocorticoid signaling through intricate interactions with steroid receptors is vital for healthy development. While natural glucocorticoids (cortisol and corticosterone) can bind to both the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) to mediate cellular outcomes, synthetic glucocorticoids such as dexamethasone act exclusively by binding to the GR. The wide range of placental adaptations to glucocorticoids are likely to be in part due to the complex nature of GR signaling, which directly or indirectly regulates up to 20% of the genome (Galon *et al.* 2002). The GR is predominantly localized to the cytoplasm, bound within the GR complex containing proteins such as Hsp90, Hsp70, Fkpb51 and c-Src. Once glucocorticoids bind to the GR, the GR complex proteins are released allowing translocation to the nucleus for gene transcription/transactivation by binding to either positive or negative glucocorticoid-responsive elements (GREs) (Cain & Cidlowski 2015).

The GR is encoded by the single gene, Nr3c1, which has at least 5 characterized splice variants (GR α , GR β , GR γ , GRA and GRP). Each of these splice variants have 8 different initiation sites (A, B, C1, C2, C3, D1, D2 and D3) resulting in up to 40 potential GR isoforms (Cain & Cidlowski 2015). Receptor isoforms regulate different downstream signaling pathways with GR β thought to largely oppose the actions of GR α -A. Furthermore, GRA and GRP lack a ligand-binding domain and so indirectly regulate glucocorticoid signaling by dimerizing with GR α -A. GR α -D isoforms are predominantly expressed in the nucleus where they can regulate gene transcription in the absence of a ligand, whereas GR α -C is thought to make cells more sensitive to GR-induced cell death (Oakley & Cidlowski 2013) by regulating downstream genes such as Bcl2l11 (Wu *et al.* 2013).

The human and guinea pig placenta express at least 8 different known GR isoforms (Saif *et al.* 2015, 2016). Multiple GR isoforms have been detected in mouse dendritic cells, but the GR isoforms in the mouse placenta are unknown. This study firstly aimed to investigate the pattern of GR isoforms in the mouse placenta of males and

females and then examine if this was affected by maternal dexamethasone exposure. Secondly, we examined the relationship between the pattern of GR isoforms and markers of placental apoptosis. Glucocorticoids induce cell death via multiple pathways and while apoptosis is a normal part of placental development (Sharp *et al.* 2010), inappropriate placental apoptosis may regulate programmed disease outcomes (Fowden *et al.* 2008). Indeed, sex-specific placental apoptosis has been recently demonstrated in sheep exposed to dexamethasone (Braun *et al.* 2015) that develop impaired endocrine function in later life. We hypothesized that maternal dexamethasone would alter the expression of GR isoforms including GR α -C and result in glucocorticoid-regulated cell death in female placentae contributing to our reported sex differences in placental growth (Cuffe *et al.* 2011).

Methods

All animal experiments were approved by the University of Queensland Animal Ethics Committee (AEC approval number SBMS/355/09) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The breeding and treatment protocol used for the current study was as described previously (Cuffe *et al.* 2011, 2015, O'Sullivan *et al.* 2013). Briefly, at E12.5, pregnant mice were surgically implanted with an osmotic minipump containing either dexamethasone ($n=10$, 1 μ g/kg/h) or saline ($n=10$, 0.9%). Animals were killed for placental collection at E14.5. Collected placentae were snap-frozen in liquid nitrogen for molecular analysis or fixed in 4% paraformaldehyde, embedded in paraffin and sectioned on a microtome for protein localization.

GR isoform protein analysis

Protein was extracted from 60 mg of placental tissue using a cytosolic fractionation buffer as described previously (Saif *et al.* 2015). 1–2 placentas were selected at random from each litter such that when possible, all 10 litters were represented ($n=8$ –12 samples per sex, per treatment). When more than one placenta was used from a litter, then data were pooled for that litter. After centrifugation, the cytoplasmic fraction was collected and the pellet was suspended in nuclear extraction buffer for nuclear protein extraction. 60 μ g of total protein was loaded into 3–8% Tris-acetate gradient polyacrylamide gels (Invitrogen, Life Technologies). Each nuclear protein extract was loaded

adjacent to its cytosolic counterpart and males and females were loaded on separate gels. A cross gel calibrator sample was loaded onto blots of the same sex so that all samples belonging to the same sex could be compared between gels. Proteins were transferred onto PVDF membranes, which were incubated with the rabbit anti-human GR total antibody (1:1500, Bethyl Laboratories, Montgomery, TX, USA, Cat no. A303-491A) as previously described (Saif *et al.* 2015), before being incubated with an anti-rabbit secondary antibody (1:2500) for 1 h. Positive control samples (human placenta, mouse kidney protein) and negative controls (pre-absorbed sample with GR control peptide supplied with antibody) were run on separate blots (data not shown). An anti-beta actin antibody was subsequently applied to each membrane (ACTB, 1:4000, Bethyl Laboratories, Cat no. A300-491A) for 1 h and densitometric analysis was performed. All receptor isoform values were normalized to ACTB expression. ACTB protein expression was not affected by sex or dexamethasone exposure although expression levels differed between nuclear and cytoplasmic fractions (data not shown). For initial analysis in saline animals, protein concentrations for each GR isoform were normalized to the total GR expression (average of GR bands from within a protein sample). This allowed comparisons between GR isoforms within a sample. Protein expression differed significantly between isoforms, therefore, for subsequent analysis comparing the effects of dexamethasone on individual isoform expression, relative protein expression was used (relative to its matched saline control).

Downstream transcript analysis

mRNA levels of genes known to be regulated by specific GR isoforms were measured by QPCR. Total RNA was extracted from placental samples (7–11 placentas per sex, per treatment-samples selected as for Western blot analysis) and reverse transcribed as previously described (Cuffe *et al.* 2012). mRNA levels of *Cpeb1* (GR α -D3), *Sgk1* and *Bcl2l11* (GR α -C), *Ier3* (all isoforms) and *Nfatc1* (GR α -A) (Lu & Cidlowski 2005) were analyzed using kqstartsybr primers from Sigma Life Science (primer pair 1 for all primer sets). In addition, mRNA levels of importin 7 (*Ipo7*, a ROS-regulated molecule important for GR nuclear translocation) and heat shock protein family A (Hsp70) member 1B (*Hspa1b*, a chaperone protein involved in regulating GR function) were measured. All qPCR reactions were analyzed using the 2^{-DDCT} method compared to the geometric mean of two validated endogenous control

genes (Rn18s and *Actb*) and normalized to the mean of the saline group of their own sex. The geometric mean of Rn18s and *Actb* was not affected by treatment or sex (saline male 16.12 $\pm$ 1.285, dexamethasone male 15.87 $\pm$ 0.6245, saline female 16.16 $\pm$ 0.4246 and dexamethasone female 16.19 $\pm$ 0.5591).

Immunohistochemistry

Evidence of apoptosis was firstly assessed using immunohistochemical staining of cleaved caspase-3. Representative midline placental sections were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol ($n=3$ per group). All slides were subjected to antigen retrieval in citrate buffer at 85°C and were immersed in 0.9% hydrogen peroxide to block endogenous peroxidase activity. Slides were blocked in 2% bovine serum albumin (BSA) and incubated with a cleaved caspase-3 primary antibody (1:200, Cell Signaling, #9661) diluted in 1% BSA or a no-antibody control for 4 h at 4°C. Slides were incubated with a biotinylated anti-rabbit secondary antibody (ABC Vectastain Elite kit, Vectorlabs, Burlingame, CA, USA) before being exposed to an avidin-biotinylated enzyme complex, stained with DAB and counterstained with hematoxylin.

Protein analysis of markers of apoptosis and oxidative stress

Protein expression of apoptotic and oxidative stress markers were measured in the cytosolic fraction collected for GR quantification. 20 μ g of total protein was loaded into 15% polyacrylamide gels and transferred to low fluorescence PVDF membranes and incubated overnight with the following apoptosis-related primary antibodies; BCL2 (1:1000, Abcam, Cat#ab32124), BAX (1:1000, Cell Signaling, Cat#2772) and cleaved caspase 3 (1:1000, Cell Signaling, Cat#9661) as well as primary antibodies for the antioxidant proteins thioredoxin reductase (Txnrd1, 1:1000, Abcam, Cat#ab124954) and glutathione peroxidase (GPX, 1:1000, Abcam, Cat#ab108427). After incubation with the primary antibody of interest, all membranes were incubated with a ACTB loading control (1:20,000, Cat# A2228, Sigma Aldrich) for 1 h. After careful washing, membranes were incubated with anti-mouse and anti-rabbit fluorescent secondary antibodies for 1 h. Membranes were then scanned using the Licor Odyssey and densitometry determined using the

supplied software. Total protein levels of the proteins of interest were normalized to the ACTB loading control.

Statistical analysis

GR isoform data are presented as median, range and % expressed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article). Through the remainder of the manuscript, all data are presented as mean $\pm$ the standard error of the mean (S.E.M.). Statistical analysis was performed using GraphPad Prism 6 for Windows. For analysis of GR isoforms, three gels per sex were analyzed with a male cross gel calibrator used for the males and a female cross gel calibrator used for the females. As a different cross gel calibrator was used for males and females, all comparisons of GR isoforms between males and females are represented as ratios to total GR content. To analyse the effects of dexamethasone on GR isoform expression, all samples were normalized to the cross run calibrator for each sex. Unpaired *t*-tests were used to analyse the sex differences in GR isoform expression and to determine the differences in GR isoform expression compared to GR α A. *T*-tests were also used to compare the effects of saline and dexamethasone exposure on GR isoform expression, gene expression, expression of other proteins and protein carbonyl concentrations. When data did not fit Gaussian distribution, nonparametric analyses were performed (Mann–Whitney test). If data had unequal variance, an unpaired *t*-test with Welch's correction was applied. When normal analysis could not be performed due to the presence of zero values, data were transformed using $X=\log(X+0.1)$ before a nonparametric test was applied. $P<0.05$ was considered significant for all results.

Results

Cytoplasmic GR expression in male and female saline-exposed (control) placentae

Western blot analysis identified eleven GR bands corresponding to eight known isoforms and three unknown immunoreactive proteins ([Fig. 1A](#)). The known isoforms identified were GR α -A (94 kDa), GR α -B (91 kDa), GR α -C (81 kDa) and GR α -D1-3 (50–55 kDa) as well as the splice variants GRA (65 kDa) and GRP (74 kDa). The three unknown bands identified were products at 69, 60 and 45 kDa, which have also been identified in other species ([Saif et al. 2015, 2016](#)). These unknown bands have not been confirmed as GR isoforms but are specific products

Figure 1

Representative Western blots of cytoplasmic (Cyto) and nuclear (Nuc) GR isoforms in placentae from saline-exposed male (open bars) and female (closed bars) mouse fetuses at embryonic day 14.5 (A). Densitometry was performed to measure the expression of each isoform for both the cytoplasmic (B) and nuclear (C) protein fraction. As protein levels of males and females were performed on different blots, data is presented as a ratio of each isoform to the total GR for each sample. Data are presented as mean $\pm$ S.E.M., $n=8-12$ placentas from 10 litters per group, * $P<0.05$ represents sex difference in GR isoform, # $P<0.05$ indicates that the GR isoform in question is different to GR α -A.

detected by this well-characterized antibody. The majority of isoforms (GR α -A, GR α -B, GR α -C, GRP, GRA, GR α -D2, GR α -D3) were expressed in the cytoplasmic fraction of

all placentae investigated; however, the GR α -D1 isoform and the protein bands corresponding to 69, 60 and 45 kDa were not always expressed (Supplementary Table 1). GR α -D1 was expressed in 50% of placentae from male fetuses but all placentae from female fetuses. The 69 kDa protein was expressed in 83% of placentae from male fetuses but not expressed in any of the placentae from female fetuses. The 60 kDa protein was expressed in the cytoplasmic protein extract in 67% of placentae from male fetuses but 100% from female fetuses. The 45 kDa GR protein was expressed in the placentae of all male fetuses but only 80% of female fetuses. When each band was expressed as a ratio to total GR levels, the most highly expressed isoform detected was the unknown 45 kDa protein, which was more highly expressed in both males and females compared to GR α -A ($P < 0.05$, Fig. 1B), whereas the 69 kDa protein was lowly expressed being significantly less than GR α -A ($P < 0.05$, Fig. 1B). The protein expression of GR α -A and GRP were approximately 2-fold higher in placentae of females compared to males ($P < 0.05$, Fig. 1B), whereas the 69 kDa protein was lower in females than males ($P < 0.05$, Fig. 1B).

Nuclear GR expression in male and female saline-exposed (control) placentae

GR α -A, GR α -B, GRP, GRA and GR α -D1 were found to be expressed in the nuclear fraction of all placentae (Supplementary Table 1). The GR α -C isoform was expressed in approximately 50% of nuclear fractions from placentae of female fetuses but only in 10% of placenta from male fetuses. In contrast, the 69 kDa protein was expressed in 16% of placenta from female fetuses but in 66% of placentae from male fetuses. The 60 kDa protein was expressed in the nuclear protein extract of all placentae from male fetuses but only 66% of placentae from female fetuses. The GR α -D2 isoform expressed in 84% of placentae in both males and females and the GR α -D3 isoform expressed in all male samples but in only 84% of female samples. Similarly, the 45 kDa protein was expressed in all placentae from male fetuses but in 84% of placentae from female fetuses. GR α -D3 was found to be the most highly expressed GR isoform in the nuclear fraction of placentae of male fetuses being significantly more highly expressed than the GR α -A isoform ($P < 0.05$, Fig. 1C). GR α -D1 was also significantly more highly expressed than the GR α -A isoform ($P < 0.05$, Fig. 1C). GR α -D3 protein levels in placentae of female fetuses were found to be approximately 50% of levels measured in placentae of male fetuses ($P = 0.06$, Fig. 1C).

The effect of maternal dexamethasone infusion on placental GR isoform patterns

Maternal dexamethasone exposure altered the expression of 6 different GR isoforms in a sex and cellular compartment-dependent manner (Fig. 2). Maternal dexamethasone exposure increased GR α -A protein levels in the cytoplasmic fraction of placentae of male (Fig. 2C, $P < 0.05$) fetuses. In contrast, GR α -C protein levels were decreased in the cytoplasmic fraction of placentae of dexamethasone-exposed male fetuses (Fig. 2C, $P < 0.05$). Maternal dexamethasone exposure had no effect on the expression levels of any GR isoform in the cytoplasmic fraction of placentae of female fetuses (Fig. 2D). Maternal dexamethasone exposure reduced the expression of nuclear GRA (Fig. 2E, $P < 0.05$) but increased the expression of GR α -D2 (Fig. 2E, $P < 0.05$). Dexamethasone-exposed placentae of female fetuses had increased levels of GR α -D1 and GR α -D3 compared to placentae from saline-exposed fetuses (Fig. 2E, $P < 0.05$). Maternal dexamethasone exposure tended to increase GR α -C expression in the nuclear fraction of placentae of female fetuses, but this did not reach statistical significance likely due to the fact that some samples in each group did not express this isoform.

The effects of maternal dexamethasone infusion on placental mRNA expression of genes regulated by specific GR isoforms

Given that the GR isoform profile was affected by maternal dexamethasone exposure in a sex-specific manner, mRNA levels of genes known to be regulated by specific GR isoforms were assessed. Dexamethasone exposure did not affect mRNA levels of *Ier3* (regulated by all GR isoforms), *Nfatc1* (regulated by GR α -A), *Bcl2l11* (regulated by GR α -C) or *Cpeb1* (regulated by GR α -D3) in placentae of male fetuses (Fig. 3). *Sgk1*, a gene known to be regulated by GR α -C, was increased by 53% in dexamethasone-exposed placentae of males compared to saline-exposed placentae of males (Fig. 3). In contrast, dexamethasone increased *Ier3* by 58%, reduced *Nfatc1* by 34% and increased *Bcl2l11* by 38% and *Sgk1* by 96% in placentae of female fetuses compared to saline-exposed placentae (Fig. 3).

As nuclear translocation of the GR is an important mediator of GR signaling, expression of two factors known to regulate this process were examined. Maternal dexamethasone exposure increased *Hspa1b* mRNA levels in placentae of male ($P < 0.05$) but not female fetuses (saline male- 1.06 ± 0.11 , dexamethasone male- 1.64 ± 0.23 , saline female- 1.04 ± 0.11 , dexamethasone female- 1.18 ± 0.12)

Figure 2

Representative Western blots of cytoplasmic (Cyto) and nuclear (Nuc) GR isoforms in saline (open bars) and dexamethasone (closed bars) exposed placentae from male (A) and female (B) mouse fetuses at embryonic day 14.5. Densitometry was performed to measure the expression of each isoform for both the cytoplasmic (C and D) and nuclear (E and F) protein fractions. All samples from each sex were measured on three blots with a common sample on each membrane. As such total isoform expression was normalized to the average expression of the saline group for each isoform. Data are presented as mean $\pm$ s.e.m., $n=8-12$ placentas from 10 litters per group, * $P<0.05$ represents significant effect of dexamethasone exposure.

and had no effect on *Ipo7* mRNA levels in either males or females (saline male- 1.21 ± 0.20 , dexamethasone male- 1.51 ± 0.26 , saline female- 1.09 ± 0.14 , dexamethasone female- 1.08 ± 0.08).

Effect of maternal dexamethasone exposure on placental apoptosis

With female placentae having lower basal levels of the anti-apoptotic GR isoform GRα-D3 and more frequently expressing the pro-apoptotic GR isoform GRα-C than males, we determined the impact of dexamethasone exposure on apoptotic factors in addition to *Bcl2l11*. This was particularly important given that dexamethasone reduced cytoplasmic expression of GRα-C in placentae of male fetuses. Cleaved caspase three immunostaining

was only observed within the placental junctional zone (Fig. 4A, B, C and D). While staining was absent in saline-exposed placentae (Fig. 4A and B), detectable staining was observed in placentae of dexamethasone-exposed male fetuses (Fig. 4C) and marked staining observed in placentae of dexamethasone-exposed female fetuses (Fig. 4D). While staining was not detected in all histological sections of placentas from male fetuses, marked staining was detected in all sections from female fetuses. Western blot analysis demonstrated that maternal dexamethasone exposure increased cleaved caspase-3 levels in placentae of both male and female fetuses (Fig. 4E and F, $P<0.05$). In placentae of males, cleaved caspase-3 levels were increased by 55% but in females, the cleaved caspase-3 was increased by more than 250% compared to placentae of saline-

Figure 3

The effects of maternal dexamethasone (closed bars) on placental mRNA expression of target genes known to be regulated by GR isoforms in males (A) and females (B) compared to saline-exposed placentae (open bars). Data are presented as mean $\pm$ S.E.M., $n=7-11$ placentas from 10 litters per group, * $P<0.05$ represents significant effect of dexamethasone exposure. *Ier3*, immediate early response 3; *Nfatc1*, nuclear factor of activated T-cells 1; *Sgk1*, serum glucocorticoid kinase 1; *Bcl2l11*, Bcl-2-like protein 11; *Cpeb1*, cytoplasmic polyadenylation element-binding protein 1. *Ier3* is a marker of regulation by any GR isoform, *Nfatc1* is regulated specifically by GR α -A, *Sgk1* and *Bcl2l11* are regulated specifically by GR α -C and *Cpeb1* is regulated by GR α -D isoforms.

exposed fetuses. Maternal dexamethasone had no effect on protein expression of BAX or BCL2 in placentae of either males (Fig. 4G and I) or females (Fig. 4H and J, $P<0.05$).

Effect of maternal dexamethasone exposure on placental reactive oxygen species and antioxidant expression

Glucocorticoid exposure can increase cellular oxidative stress, which can indirectly induce apoptosis. As such, this study measured placental protein carbonyl concentrations as a marker of oxidative stress, as well as protein levels of key antioxidants GPX1 and TXNRD1. Maternal dexamethasone exposure had no effect on placental protein carbonyl levels in males or females (Fig. 5A and B). Dexamethasone exposure increased protein expression of GPX and TXNRD1 in the placentae of males (Fig. 5C and E) but not females (Fig. 5D and F).

Discussion

This study has for the first time demonstrated the presence of multiple GR isoforms in the mouse placenta with eight known isoforms identified. In addition, we have identified three unknown proteins with GR immunoreactivity of similar size to those previously reported (Saif *et al.* 2015, 2016). Importantly, placentae of male and female mice have different GR isoform profiles that likely confer sex-specific cellular sensitivity to glucocorticoids. Of particular interest, nuclear expression of the anti-apoptotic isoform GR α -D3 was greater in placentae of males compared to

females while nuclear expression of the pro-apoptotic isoform GR α -C was higher in female placentae. This suggests that differences in placental glucocorticoid sensitivity between the sexes may be mediated by the differential expression of GR α -D and GR α -C isoforms and that apoptosis is likely to play a greater role in mediating the placental adaptations to glucocorticoid exposure in females than those in males.

The GR isoform profile of the murine placenta was similar to that in placentae of humans (8 known and five unknown) and guinea pigs (8 known and two unknown) (Saif *et al.* 2015, 2016), indicating analogous functional regulation of glucocorticoids between species. Indeed, using the same GR antibody, Orgeig and coworkers demonstrated that placental restriction in sheep reduces the expression of multiple GR isoforms in the fetal lung (Orgeig *et al.* 2015). Although GR was not measured in the placentas of these sheep, marked cellular and functional changes were noted in the placenta (Zhang *et al.* 2016). In our study in mice, GR α -C expression within the nuclear compartment was sex specific as was the expression of cytoplasmic isoforms such as GR α -A and GRP. In humans, placentae of male and female babies demonstrated sex-specific changes in GR α -C when born preterm compared to term while betamethasone exposure in guinea pigs also changed GR isoforms in a sex-specific manner. Despite similarities, there were key differences in GR isoform patterns between species. For example, GR α -C was not expressed at all in placentae of guinea pigs and instead other GR isoforms displayed sex-specific upregulation by glucocorticoid exposure (Saif *et al.* 2016). The most dominant isoform expressed also differed between species with GR α -D1 being the most dominant isoform in the human placentae (Saif *et al.* 2015), but the 45 kDa protein being the most highly expressed within the cytoplasmic compartment and GR α -D3 the most highly expressed within the nucleus in mice.

Maternal dexamethasone exposure was also shown to affect GR isoform patterns within the placenta differentially in the cytoplasm and nucleus. The current study demonstrated that dexamethasone increased the expression of GR α -A, but reduced the expression of GR α -C in the cytoplasmic fraction of the placental extracts of males but not females. These changes in GR isoform expression may mediate a range of non-genomic activities including regulation of chaperone proteins and altering cell signaling by interacting with MAPK/ERK/AKT pathways (Mitre-Aguilar *et al.* 2015). Indeed, these glucocorticoid-induced changes in GR isoform expression levels may have contributed to the sex-specific regulation

Figure 4

Immunohistochemical staining of cleaved caspase three (brown DAB pigment) in placentae of male (A and C) and female fetuses (B and D) exposed to saline (A and B) or dexamethasone (C and D) during pregnancy ($n=3$ per group). Isotype control sections are presented as inset images. All slides are counterstained with hematoxylin. The effects of maternal dexamethasone (closed bars) on placental cytoplasmic protein expression of cleaved caspase three in males (E) and females (F), BAX in males (G) and females (H) and BCL2 in males (I) and females (J) compared to saline-exposed placentae (open bars). Data is presented as mean $\pm$ S.E.M., * $P<0.05$ represents significant effect of dexamethasone exposure. Representative blots contain $n=6$ out of the $n=8-12$ placentas from 10 litters per group analyzed.

of MAPK previously demonstrated (Cuffe *et al.* 2011). In contrast, dexamethasone exposure had no effect on the expression of any GR isoforms within the cytoplasm of placentae of female fetuses suggesting that glucocorticoids

may predominantly affect females through nuclear regulation of gene expression.

Nuclear expression of the 48 kDa protein was increased with betamethasone treatment in human female

Figure 5

The effects of maternal dexamethasone (closed bars) on placental protein carbonyl levels in males (A) and females (B), placental GPX protein concentrations in males (C) and females (D) and TXNRD1 protein concentrations in males (E) and females (F) compared to saline-exposed placentae (open bars). Data is presented as mean $\pm$ S.E.M., * $P < 0.05$ represents significant effect of dexamethasone exposure. Representative blots contain $n = 6$. $n = 8-12$ placentas from 10 litters per group were analyzed for Western blotting and protein carbonyl ELISAs.

placentae but no others isoforms were affected in either sex (Saif *et al.* 2015). In guinea pig, preterm placentae exposed to betamethasone, there was an increase in GR α -A and GR β in both sexes but male placentae had an increase in GR α -D2 and D3 (Saif *et al.* 2016). The nuclear expression of GR isoforms in response to dexamethasone exposure in murine placental tissue is more complex with GRA being reduced and GR α -D2 being increased in males while GR α -D1 and 3 were increased in females. Each of these isoforms can function in the absence of ligand suggesting that these glucocorticoid-induced changes in nuclear GR isoform expression are not due to nuclear translocation but through other mechanisms. Furthermore, the transactivational activity of the GR α -D isoforms is lower than other GR isoforms suggesting that dexamethasone exposure has reduced glucocorticoid sensitivity by altering the expression of these isoforms. The cellular mechanisms responsible for dexamethasone-regulated GR isoform patterns are unclear but likely to be complex. Buoso and coworkers demonstrated that cortisol alters spliceosome proteins SRSF3 and SRSF9, which regulate the GR splice variants that are produced

(Buoso *et al.* 2017). This suggests that the dexamethasone-induced regulation of the GR splice variant GRA in the current study may be due to altered spliceosomal activity. In addition, isoforms may have been affected by glucocorticoid-induced regulation of GR translation. Lu and Cidlowski previously demonstrated that ribosomal leaky scanning is responsible for GR α -B and C production, while ribosomal shunting is involved in GR α -C and D formation (Lu & Cidlowski 2005). These processes may be regulated by epigenetic mechanisms with transcriptional microvariability thought to contribute to GR translational isoform profiles (Leenen *et al.* 2016). To date, no studies have investigated if glucocorticoids influence these processes but given our findings of changed GR α -C and D expression, these translational mechanisms may be involved.

Sex differences in glucocorticoid responsivity can regulate sex-biased gene expression profiles. A recent review proposes that glucocorticoid-induced GR expression in the placenta may mediate these gene profiles to affect fetal growth (Bivol *et al.* 2016). In mice, sex differences in GR levels mediate the dimorphic expression of inflammatory

genes within the liver and deletion of the GR eliminated these sex differences (Quinn & Cidlowski 2016). Differences in GR isoforms in dendritic cells have been shown to be responsible for the distinct gene profiles that change with maturity (Cao *et al.* 2013). Different populations of helper T-cells (TH1, TH2, TH17) express different GR isoforms, which determine GR sensitivity and regulate different patterns of glucocorticoid mediated gene expression (Banuelos & Lu 2016). To date, these studies have highlighted the key differences in GR isoform regulation of apoptosis but there are undoubtedly other less well-characterized pathways affected in a sex- and tissue-specific manner.

Genes known to be regulated by different GR isoforms were similarly regulated by dexamethasone in a sex-specific manner in the mouse placenta. *Ier3* (regulated by all GR isoforms) and *Nfatc1* (GR α -A) were both affected by dexamethasone exposure in placentae from females but not males. Perhaps surprisingly, given that dexamethasone increased GR α -D expression in both sexes, *Cpeb1* (GR α -D) mRNA levels were unaffected by dexamethasone in either sex. This might suggest that either *Cpeb1* is not regulated by GR α -D isoforms in placental cells or that the limited transactivational activity of GR α -D protein prevented significant regulation of *Cpeb1* expression. *Sgk1* (GR α -C) was increased by dexamethasone but was increased to a much greater extent in females. This greater increase in *Sgk1* further supports a more dominant role of GR α -C in the placental adaptations to glucocorticoids in females compared to males. Jurak cells transfected with GR α -C have been shown to be highly vulnerable to dexamethasone-induced cell death (Wu *et al.* 2013) and the fact that GR α -C was only expressed in females in the current study is likely to have significantly influenced the regulation of apoptotic pathways. Indeed, *Bcl2l11* has been shown to be regulated predominantly by GR α -C and in the current study was increased in the placentae of females but not males. *Hspa1b*, encodes HSP70, which along with HSP90 is an important component of the GR complex. While HSP90 promotes recovery of ligand binding, HSP70 inactivates the GR by partially unfolding the GR ligand-binding domain (Kirschke *et al.* 2014). Interestingly, *Hspa1b* mRNA levels were increased by 55% in dexamethasone-exposed placentae of male fetuses but did not change in placentae of female fetuses. This may further explain the observed increase in GR insensitivity in males compared to females and the glucocorticoid-induced increase in GR α -A in the cytoplasmic compartment.

In our model, maternal dexamethasone administration lowers placental weight in female fetuses only (Cuffe *et al.* 2011). Morphological assessment demonstrated this was due to a reduction in the junctional zone area in females, a common placental adaptation to maternal stress (Coan *et al.* 2010, Mark *et al.* 2011). In response to a maternal challenge, the junctional zone has been shown to decrease in size to allow for expansion of the labyrinth (Burton & Fowden 2012). The current study demonstrated cleaved caspase-3 staining only within the junctional zone. Western blotting analysis demonstrated that dexamethasone exposure increased cleaved caspase-3 levels in both sexes; however, the increase was much greater in females. Previous studies have demonstrated that maternal dexamethasone exposure leads to apoptosis within the junctional zone of the rat placenta (Waddell *et al.* 2000, Ain *et al.* 2005); however, these studies did not investigate placentae of males and females separately. Apoptosis is also known to be initiated by oxidative stress. Restraint stress has been shown to increase oxidative stress levels in neurological tissues through endogenously produced glucocorticoids (Spiers *et al.* 2016). Furthermore, oxidative stress plays a key role in the etiology of many placental-related disorders (Jauniaux *et al.* 2006). We thus determined whether the apoptosis in the current study was a result of oxidative stress rather than direct GR signaling. This is of key interest given the GR is able to regulate expression of nuclear-encoded mitochondrial genes (*Pgc1a* and *Nrf1*) and activation of cytosolic GR receptors affect mitochondrial function (Lee *et al.* 2013). We found placental protein carbonyl levels were not affected by dexamethasone exposure in the mouse. Protein carbonyl levels are routinely used as markers of oxidative stress and are elevated in placental tissue of asthmatic women (Clifton *et al.* 2005). A range of antioxidants, including TXNRD1 and GPX, were also increased in asthmatic women independent of fetal sex. In the current study, TXNRD1 and GPX protein levels were increased in placentae of male but not female dexamethasone-exposed fetuses. This indicates that placentae of male fetuses may have additional glucocorticoid-regulated protective mechanisms against oxidative stress induced apoptosis in support of continued placental growth.

Conclusion

This study has for the first time demonstrated multiple GR isoforms in murine placenta. The pattern of GR

isoforms expressed with the placenta is dependent on fetal sex and is regulated by exposure to glucocorticoids. Placentae of female fetuses were found to have greater levels of GR α -C and lower GR α -D, which may enhance the sensitivity to glucocorticoids and increase the degree of placental apoptosis compared to males. Importantly, however, this apoptosis was only increased within the junctional zone of placentae from female fetuses indicating that female fetuses are able to prioritize the labyrinth zone at this stage of gestation in response to a maternal challenge. Placentae of male fetuses continue to maximize their growth throughout this period of stress through the expression of the GR α -D isoforms and via an alteration in GR protein configuration via HSP70 that reduces ligand binding. Apoptotic regulated reduction in junctional zone size in females may result in improved management of fetal resources which in turn contributes to the improved outcomes in later life for the female.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-17-0171>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J S M C designed project, generated animals, performed most laboratory work and wrote most of the manuscript. Z S and J S M C performed GR isoform Western blots. A V P assisted with oxidative stress and antioxidant assessment and manuscript editing. K M M and V L C assisted with project design and manuscript editing.

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