Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway

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Abstract

Intrauterine growth restriction (IUGR) is a major cause of perinatal death and neonatal morbidity and mortality. There are numerous causes of IUGR. Glucocorticoid-induced IUGR is highly relevant because administration of synthetic glucocorticoids, principally dexamethasone, to women threatened by premature labor is widely used in clinical practice. Fetal growth is directly related to placental growth and development. In this report, we analyzed the effect of dexamethasone on placental development in the rat. Dexamethasone administered between days 13 and 20 of pregnancy not only induced IUGR but also decreased placental mass by approximately 50%. Impaired placental development was associated with dys-regulated placental prolactin (PRL) family and insulin-like growth factor-II (IGF-II) gene expression. Furthermore,

there was a significant decrease in the activation of Akt/protein kinase B in the junctional zone of the placenta, as assessed by the phosphorylation status of Akt and the pro-apoptotic protein BAD, a downstream target of the Akt signaling pathway. Such changes are consistent with increases in indices of apoptosis, including increased cleavage of poly(ADP-ribose) polymerase (PARP) in the junctional zone of the placenta of dexamethasone-treated rats. In summary, dexamethasone-induced IUGR is associated with placental insufficiency, including dys-regulated placental hormone/cytokine gene expression and down-regulation of the IGF-II/Akt signaling pathway resulting in increases in indices of placental apoptosis.

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Introduction

The placenta is an extraembryonic tissue that is situated between maternal and fetal compartments and acts to ensure the normal progression of fetal development. This task is achieved through regulating nutrient and waste transport and modulating the maternal environment through the elaboration of an assortment of hormones, growth factors, and other regulatory molecules. Understanding mechanisms underlying the growth and development of the placenta is paramount to any appreciation of fetal development.

The rat chorioallantoic placenta is organized into two compartments: the junctional zone and the labyrinth zone. The junctional zone is comprised of stem cells and three differentiated cell types: (i) trophoblast giant cells, (ii) spongiotrophoblast cells, and (iii) glycogen cells. Trophoblast giant cells arise by endoreduplication, are situated at the maternal–placental interface, and are one of the major endocrine cells of the placenta (Soares *et al.* 1996). They synthesize and secrete steroid and peptide hormones. Spongiotrophoblast cells are located immediately beneath

the trophoblast giant cell layer and synthesize and secrete peptide hormones. Glycogen cells are a transient cell type embedded among the spongiotrophoblast cells. Beyond their accumulation of glycogen, their biology is not well understood. The labyrinth zone is located at the fetal interface and is comprised of stem cells capable of differentiating into trophoblast giant cells or fusing to form syncytial trophoblast cells. The labyrinthine trophoblast giant cells possess a restricted capacity for hormone production (Soares *et al.* 1996), whereas syncytial trophoblast cells mediate the transfer of nutrients and wastes between maternal and fetal compartments (Knipp *et al.* 1999). The composition of the trophoblastic elements within each zone changes through pregnancy. Each trophoblast lineage of the placenta develops specialized functions required for successful pregnancy. Disruptions in trophoblast development can lead to early pregnancy loss or intrauterine growth restriction (IUGR). These represent serious health problems whose etiologies are not sufficiently understood. Differentiation of trophoblast cells can be monitored by their endocrine activities, which include expression of members of the prolactin (PRL) gene family

(Dai *et al.* 2002). PRL gene family members have been implicated for their roles in the regulation of maternal adaptation to pregnancy (Soares & Linzer 2001, Soares 2004).

Synthetic glucocorticoids, including dexamethasone, have been used for over three decades to mature fetal lungs and prevent respiratory distress syndrome in pregnancies where delivery is likely before 34 weeks (Liggins & Howie 1972, Trainer 2002). Although glucocorticoids promote lung maturation, these actions are not without negative side effects. Exposure to glucocorticoids retards fetal growth in animal models and in humans (Seckl 1994, Gluckman 2001). In rats, fetal-placental exposure to maternally administered glucocorticoids decreases birth weight and placental weight (Benediktsson *et al.* 1993, Sugden & Langdown 2001, McDonald *et al.* 2003). The mechanism underlying the placental growth inhibitory effects of dexamethasone have not been elucidated.

Insulin-like growth factor (IGF)-II is a potential target for glucocorticoid actions on placental development. Evidence exists that glucocorticoids regulate IGF-II expression in a variety of cell types and tissues using both *in vitro* and *in vivo* models (Levinovitz & Norstedt 1989, Cheng *et al.* 1998, Smink *et al.* 2002) and IGF-II is viewed as a key autocrine/paracrine placental growth factor (DeChiara *et al.* 1990, Barker *et al.* 1993, Constanica *et al.* 2002). In the mouse, IGF-II null mutations lead to significant IUGR of both the fetus and placenta (DeChiara *et al.* 1990, Lopez *et al.* 1996). The rodent placenta possesses all the components of the IGF-II signaling pathway (Zhou & Bondy 1992, Barker *et al.* 1993, Lopez *et al.* 1996).

Both IGF-I and IGF-II act on their target cells via interactions and activation of the IGF receptor type I (IGFR-I) (Nakae *et al.* 2001). Phosphatidylinositol 3-kinase (PI3-kinase) signaling pathway is a downstream effector of IGFR-I receptor signaling (Kulik & Weber 1998, Moorehead *et al.* 2001). PI3-kinase signals through several pleckstrin homology domain containing regulatory proteins, including the Ser/Thr protein kinase, Akt (also called protein kinase B) (Chan *et al.* 1999, Datta *et al.* 1999). Akt activation can stimulate changes in gene transcription, cell survival, cell division, and cell differentiation.

In the present report we examined the effects of dexamethasone on the placental phenotype and demonstrate that compromised placental growth following dexamethasone treatment is associated with dysregulated placental PRL family gene expression and down-regulation of the IGF-II/Akt signaling pathway leading to increases in indices of placental apoptosis.

Materials and Methods

Animals and tissue preparation

Holtzman rats were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA). To obtain timed

pregnancies, females were caged overnight with fertile males. The presence of sperm in the vaginal smear was designated as day 0 of pregnancy. On day 13 of pregnancy, pregnant rats were subcutaneously injected with a bolus dose of 100 µg dexamethasone acetate (Sigma, St Louis, MO, USA) in 0.1 ml 10% ethanol. Another group of gestation day 13 pregnant rats was injected with the vehicle only and used as controls. Animals were then anesthetized with halothane, and an alzet osmotic pump (model # 2 ML1, Durect Corp., Cupertino, CA, USA) was subcutaneously implanted. The osmotic pumps were calibrated to release 200 µg dexamethasone acetate/kg maternal body weight/day. Control rats received osmotic pumps containing vehicle. Four animals were used in each group. Animals were killed on day 20 of pregnancy. We selected this route and dose of dexamethasone treatment because it was previously demonstrated to cause IUGR in the rat (Benediktsson *et al.* 1993, Levitt *et al.* 1996, Sugden & Langdown 2001). Placental tissues (junctional zone and labyrinth zone) were dissected from pregnant animals. Tissues were snap-frozen in liquid nitrogen for PRL family mini-array and Northern and Western analysis. For *in situ* hybridization, tissues were frozen in dry ice-cooled heptane. All tissue samples were stored at -80 °C until used. The University of Kansas Medical Center Animal Care and Use Committee approved all procedures for handling and experimentation with rodents.

PRL family mini-array assay

The PRL family mini-array assay, a hybridization-based tool for simultaneously monitoring expression of each member of the rat PRL family (Dai *et al.* 2002), was used to monitor trophoblast endocrine function. The PRL family mini-array assay was performed as previously described (Dai *et al.* 2002, Ain *et al.* 2003). Twenty nanograms PCR-amplified cDNA for each of the members of the rat PRL family were spotted, in duplicate, onto nylon membranes. Membranes were crosslinked and stored at 4 °C until used. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). [α P³²]dCTP-labeled cDNA probes were generated by reverse transcription using 5 µg total RNA. Total RNA was isolated from two pooled and randomly selected placentas per uterus from each animal. A total of four samples were analyzed per treatment group. Probes were purified using micro bio-spin columns (Bio-Rad Laboratories, Richmond, CA, USA). Membrane filters were briefly rinsed with water and pre-hybridized for 2 h at 42 °C with 5 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, 10 mM EDTA, pH 7.4) containing 5 × Denhardt's reagent, 50% deionized formamide, 1% SDS, and salmon sperm DNA (100 µg/ml). Hybridizations were performed overnight with the labeled probes at 42 °C. Membranes were washed once with 2 × SSPE and 0.1% SDS for 30 min at 42 °C and twice with

0.1 × SSPE and 0.5% SDS at 60 °C for 30 min each. Membranes were then wrapped with plastic wrap and exposed to Kodak Bio-Max film (Kodak, Rochester, NY, USA) for 1–4 h and developed.

Northern blot analysis

Northern blot analysis was performed as described previously (Faria *et al.* 1990). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen). Total RNA (20 µg/lane) was resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes, and crosslinked. Blots were probed with α P³²-labeled cDNAs for PRL-like protein-B (PLP-B), PLP-C, placental lactogen-Iv (PL-Iv), PL-II (Dai *et al.* 2002) and IGF-II. Glyceraldehyde-3'-phosphate dehydrogenase (G3 PDH) cDNA was used to evaluate the integrity and equal loading of RNA samples. At least three different tissue samples from three different animals were analyzed with each probe.

In situ hybridization

In situ hybridization was performed as described previously (Ain *et al.* 2003, 2004). Ten-micron cryosections of tissues were prepared and stored at –80 °C until used. Plasmids containing cDNAs for rat PLP-C, PL-II and IGF-II were used as templates to synthesize sense and anti-sense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). Tissue sections were air dried and fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Pre-hybridization was carried out in a humidified chamber at 50 °C in 5 × SSC (standard saline citrate), 50% deionized formamide, 1 × Denhardt's reagent, 10% dextran sulfate and salmon sperm DNA (100 µg/ml). Hybridizations were performed in the same incubation conditions overnight. Slides were washed in 2 × SSC at room temperature for 30 min followed by treatment with RNase-A (100 ng/ml) and additional washes with 2 × SSC for 30 min at room temperature, 2 × SSC for 1 h at 65 °C, and 0.1 × SSC for 1 h at 65 °C. Tissue samples were then blocked for 30 min and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500) in blocking buffer (Roche Molecular Biochemicals) for 2 h at room temperature. Slides were then washed and detection was performed using nitro blue tetrazolium (250 µg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (225 µg/ml; Roche Molecular Biochemicals).

Western blot analysis

Tissues were homogenized in a Tris saline buffer (50 mM Tris, 150 mM NaCl, pH 7.4, containing 1% NP-40, 1 mM EDTA, 0.1 mg/ml PMSF, and a protease inhibitor

cocktail; Sigma). Samples were then centrifuged and the supernatants collected. Protein concentrations were determined for each sample using the Bio-Rad DC protein assay (Bio-Rad). Proteins were separated by SDS-PAGE under reducing conditions and were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The blots were then incubated for one hour in blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, TBST) and then overnight with rabbit polyclonal antibodies obtained from Cell Signaling (Beverly, MA, USA) to phosphorylated-Akt (Ser⁴⁷³, cat. no. 9271), Akt (cat. no. 9272), phosphorylated-BAD (Ser¹³⁶, cat. no. 9295), BAD (cat. no. 9292), or poly(ADP-ribose) polymerase (PARP, cat. no. 9542). Antibodies were diluted to 1:1000 either in blocking solution or in TBST containing 5% bovine serum albumin (BSA). After washing the membranes in TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling) diluted 1:2000 in blocking solution for one hour at room temperature. After washing with TBST, bound antibodies were detected by chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were then stripped using stripping buffer (Pierce Chemical Co., Rockford, IL, USA) and re-probed using β -actin antibody (Sigma) using the above protocol.

Statistical analysis

The data were analyzed by analysis of variance. The source of variation from significant F ratios was determined with the Newman-Keuls multiple comparison test (Keppel 1973).

Results

Maternal dexamethasone administration influences fetal and placental development and placental hormone production

Similar to previous reports, dexamethasone administration during the last third of gestation resulted in both fetal (Fig. 1A, $P < 0.01$) and placental growth restriction (Fig. 1B, $P < 0.01$, and Fig. 1C). Additionally, maternal body weight gain was affected by dexamethasone treatment (control, 59 ± 7 g versus dexamethasone, 34 ± 3 g, $P < 0.01$). Dexamethasone treatment did not affect litter size (number of fetuses) or fetal viability.

The junctional zone of the chorioallantoic placenta is the primary source of members of the PRL family of hormones/cytokines during the last week of pregnancy, whereas the labyrinth zone makes a lesser contribution. The PRL family mini-array hybridization assay was used to survey placental responses to maternal dexamethasone exposure. Maternal dexamethasone treatment resulted in the dysregulation of PRL family gene expression in both

Figure 1 Effects of dexamethasone (Dex) on fetal and placental development. Fetuses and placentas were dissected on day 20 of gestation from control and Dex-treated rats. (A) Fetal weights decreased by 23% in the Dex-treated group as compared with control ($P<0.01$). (B) Placental weights decreased by 51% in the Dex-treated group as compared with control ($P<0.01$). (C) Hematoxylin and eosin staining of gestation day 20 placentas from control and Dex-treated animals. Scale bars, 1 mm.

regions of the chorioallantoic placenta (Fig. 2). In general, if affected, PRL family gene expression was inhibited in the junctional zone, whereas PRL family gene expression was stimulated in the labyrinth zone. The inhibitory effect of dexamethasone on junctional zone gene expression was best exemplified by monitoring PLP-B mRNA levels, whereas in the labyrinth zone PLP-C mRNA showed the most dramatic response (Fig. 2). Northern blot analyses were used to further validate the expression of the PRL gene family in the placenta (Fig. 3A). Maternal dexamethasone treatment dramatically inhibited junctional zone PLP-B transcripts. PL-II showed modest changes in either placental zone following dexamethasone treatment (Fig. 3A, B and C). The influence of dexamethasone on the expression of PLP-C transcripts was dependent on the placental zone. Dexamethasone treatment had minimal effects on PLP-C mRNA levels in the junctional zone, in

contrast to a dramatic upregulation of labyrinthine PLP-C mRNA levels (Figs 2 and 3).

The expression of PLP-C in the labyrinth zone is an aberration, which prompted an investigation of the cellular site of PLP-C expression. Tissue distributions of PLP-C mRNAs were monitored by *in situ* hybridization (Fig. 3D). Expression of PL-II was used as a control as its expression was not substantially altered by dexamethasone treatment and is restricted to trophoblast giant cells of the junctional and labyrinth zones. PLP-C transcripts were not detectable by *in situ* hybridization in the labyrinth zone of the placenta from the control group. Maternal dexamethasone treatment resulted in the detection of colonies of PLP-C mRNA-positive cells in the labyrinth zone. Morphologically, the cells in these colonies resembled spongiotrophoblast cells of the junctional zone. In summary, maternal dexamethasone

Figure 2 Impact of *in vivo* dexamethasone (Dex) treatment on PRL gene family expression in placentas from gestation day 20. (A) Representative PRL family mini-array analyses of RNAs isolated from the junctional and labyrinth zones of the chorioallantoic placenta. Total RNA samples were isolated on day 20 of gestation, radiolabeled by reverse-transcription, and used as hybridization probes for rat PRL family mini-arrays. Glyceraldehyde-3'-phosphate dehydrogenase (G3 PDH) was used as control. Additional abbreviations: PRL, prolactin; GH, growth hormone; PL, placental lactogen; PLF-RP, proliferin-related protein; PLP, PRL-like protein; d/tPRP, decidual/trophoblast PRL-related protein. (B and C) Densitometric analyses of PRL family mini-arrays (based on 3–4 replicate experiments) for selected PRL family genes - (B) junctional zone, (C) labyrinth zone.

affected placental development and hormone/cytokine gene expression.

Dexamethasone regulates IGF-II mRNA expression by the placenta

In the next series of experiments, we sought to account for the intrauterine placental growth retardation. Initially, we examined the effect of maternal dexamethasone treatment on placental IGF-II expression. Northern blot analysis showed that there was a prominent decrease in IGF-II mRNA concentrations in junctional zones from dexamethasone-treated animals (Fig. 4A and B). Maternal dexamethasone treatment had only modest effects on

IGF-II expression in the labyrinth zone (Fig. 4A, B). *In situ* hybridization of placental IGF-II mRNA expression corroborated the Northern blot analysis (Fig. 4C). IGF-II signaling is critical to placental development (DeChiara *et al.* 1990, Barker *et al.* 1993, Constancia *et al.* 2002). Thus, maternal dexamethasone-associated placental growth restriction may be mediated, at least in part, by decreased placental IGF-II.

Dexamethasone down-regulates Akt signaling pathway and increases indices of placental apoptosis

IGFs act, at least in part, through the PI3-kinase/Akt signaling pathway (Kulik & Weber 1998, Moorehead *et al.* 2001).

Figure 3 Expression of mRNAs for PL-Iv, PLP-B, PLP-C and PL-II in placentas from dexamethasone (Dex)-treated and control rats. (A) Northern blot analysis using total RNA isolated from gestational day 20 junctional and labyrinth zones of chorioallantoic placentas from control and Dex-treated groups. RNAs were fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon, and hybridized to ³²P-labeled cDNA probes for PL-Iv, PLP-B, PLP-C, PL-II or G3 PDH. G3 PDH was used to evaluate the integrity of the RNA samples and as a control for gel loading. (B and C) Densitometric analyses of mRNA levels - (B) junctional zone, (C) labyrinth zone. (D) *In situ* localization of PLP-C and PL-II mRNAs in placentas from control and dexamethasone-treated rats. Ten-micron cryosections of the tissues were prepared and hybridized to digoxigenin-labeled anti-sense and sense (data not shown) probes for PLP-C and PL-II. Green arrowheads indicate PLP-C positive cells in the labyrinth zone. Scale bars, 250 µm. JZ, junctional zone; LZ, labyrinth zone.

Figure 4 Expression of IGF-II mRNA in placentas from dexamethasone (Dex)-treated and control rats. (A) Northern blot analysis using total RNA isolated from gestational day 20 junctional and labyrinth zones of chorioallantoic placentas from control and dexamethasone-treated groups. RNA was fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon, and hybridized to ^{32}P -labeled cDNA probes for IGF-II or G3 PDH. G3 PDH was used to evaluate the integrity of the RNA samples and as a control for gel loading. (B) Densitometric analyses of IGF-II mRNA levels. (C) *In situ* localization of IGF-II mRNA in rat placentas from control and dexamethasone-treated groups. Ten-micron cryosections of the tissues were prepared and hybridized to digoxigenin-labeled anti-sense and sense (data not shown) probes for IGF-II. Scale bars, 250 μm . JZ, junctional zone; LZ, labyrinth zone.

Thus, we examined the effects of maternally administered dexamethasone on placental Akt signaling. Junctional zone lysates from control and dexamethasone-treated groups were subjected to immunoblot analysis. Activation of the Akt pathway is associated with phosphorylation of Akt on Ser⁴⁷³. Maternal dexamethasone treatment led to a significant decrease in Akt activation (Fig. 5A and B). Total Akt protein expression was not significantly affected by the treatment (Fig. 5A). We next examined a downstream effector of Akt, BAD, a known regulator of apoptosis. Phosphorylation of BAD on Ser¹³⁶ by activated Akt prevents apoptosis. Consistent with the deactivation of Akt, we observed that maternal dexamethasone treatment also resulted in decreased phosphorylated BAD without affecting total BAD protein (Fig. 5C and D). These results suggest that pathways preventing apoptosis in the junctional zone might be disrupted in the dexamethasone-exposed placentas. Thus, we monitored the integrity of the junctional zone using cleaved PARP as a measure of apoptosis (Lazebnik *et al.* 1994). As shown in Fig. 5E and F, dexamethasone treatment was associated with an increased accumulation of the 89-kDa PARP cleavage product when compared

with controls. Blots were stripped and re-probed with β -actin to show that equal amounts of protein were loaded in each lane (Fig. 5E). Maternally administered dexamethasone leads to attenuated placental Akt signaling and increases in at least one measure of placental apoptosis, PARP cleavage.

Discussion

In most species, size of the fetus is proportional to placental size. When the size of the placenta is restricted, as in maternal malnutrition or compromised placental blood flow, the fetus is also often growth restricted (Price *et al.* 1992). A poorly developed or inefficiently functioning placenta is associated with a reduction in birth weight. In this report, we investigated the effect of maternally administered dexamethasone on placental development and function. We chose a dose and route of dexamethasone administration that reproducibly induces IUGR in rats (Benediktsson *et al.* 1993, Sugden & Langdown 2001, McDonald *et al.* 2003).

Pregnancy-dependent adjustments in maternal and fetal compartments are orchestrated by the endocrine function

Figure 5 Dexamethasone (Dex) treatment decreases Akt and BAD phosphorylation and increases cleavage of PARP. Western blot analysis of phospho (p)-Akt and Akt (A), phospho (p)-BAD and BAD (C) in junctional zone placental tissues from control and Dex-treated animals. Thirty micrograms total protein were loaded in each lane. Lanes 1–3, junctional zone samples from three different control rats; lanes 4–6, junctional zone samples from three different Dex-treated rats. All samples were harvested on day 20 of gestation. Densitometric measurements for phospho-Akt/Akt are presented in (B) and for phospho-BAD/BAD in (D). (E) Western blot analysis of PARP in junctional zone placental tissues from control and Dex-treated animals. Twenty micrograms total protein were loaded in each lane. Lanes 1–3, samples from three different control rats; lanes 4–6, samples from three different Dex-treated rats. All samples were harvested on day 20 of gestation. Blots were stripped and re-probed with β -actin antibody to ensure equal loading. (F) Densitometric measurements for the PARP and β -actin Western blotting.

of trophoblast cells (Soares *et al.* 1996). As trophoblast cells differentiate they acquire the capacity to produce hormones, cytokines, and growth factors, including members of the PRL family (Soares & Linzer 2001, Soares 2004). Maternal dexamethasone treatment resulted in the dysregulation of PRL family gene expression and compromised placental development. Dexamethasone negatively impacted production of members of the PRL family associated with the junctional zone, especially the spongiotrophoblast-specific PLP-B mRNAs. In contrast, the effect of dexamethasone on the labyrinth zone was highlighted by a marked increase in expression of PLP-C.

PLP-C is not normally expressed in the labyrinth zone (Dai *et al.* 2002). Labyrinthine PLP-C was attributed to spongiotrophoblast-like cellular colonies inappropriately developing in the labyrinth zone of the dexamethasone-treated rats. PRL family hormones/cytokines participate in the regulation of maternal and fetal adaptations to pregnancy (Soares & Linzer 2001, Soares 2004). Whether dysregulated PRL family gene expression patterns contribute directly or indirectly to the placental and/or fetal IUGR remain to be determined.

Disruptions in spongiotrophoblast cell development may be central to the placental endocrine phenotype induced

by maternal dexamethasone treatment. Interestingly, dysmorphic development of spongiotrophoblast cells is also a characteristic of interspecies hybrid and cloned placentas (Rogers & Dawson 1970, Zechner *et al.* 1996, 1997, Kurz *et al.* 1999, Tanaka *et al.* 2001). Abnormalities in genomic imprinting are implicated in these placental pathologies (Zechner *et al.* 2002, Schutt *et al.* 2003, Ohgane *et al.* 2004, Shi *et al.* 2004, Singh *et al.* 2004). There is some evidence for imprinting among members of the placental PRL family in *Peromyscus* (Vrana *et al.* 2001); however, experimentation on imprinting within the rat PRL family locus has not been reported. Imprinting is fundamental to the regulation of IGF-II, which was also affected by maternal dexamethasone treatment in our study. IGF-II is one of many genes exhibiting allele-specific expression with an impact on placental development. A more generalized influence of maternal dexamethasone treatment on the process of genomic imprinting in the developing placenta and fetus has not been reported.

The effect of dexamethasone on placental IGF-II expression provides for a logical explanation of the IUGR. Our data showed that maternal dexamethasone-induced IUGR was associated with decreased expression of IGF-II by the junctional zone of the placenta. IGF-II null mutations in mice are known to cause fetal and placental growth restriction (DeChiara *et al.* 1990).

IGFs affect cellular function, at least in part, through the PI3-kinase pathway (Kulik & Weber 1998, Moorehead *et al.* 2001). Akt is one of the principal downstream mediators of PI3-kinase action (Datta *et al.* 1999, Richards 2001, Downward 2004). We observed that maternal dexamethasone treatment was associated with decreased placental Akt activation. Of the three Akt family members, Akt1 is most abundantly expressed in the placenta (Kamei *et al.* 2002, Yang *et al.* 2003). Akt1 null mice are growth restricted due, in part, to placental insufficiency (Chen *et al.* 2001, Cho *et al.* 2001, Yang *et al.* 2003). Our findings are also consistent with earlier studies showing that the PI3-kinase/Akt pathway modulates trophoblast cell endocrine differentiation, including expression of the PRL gene family (Kamei *et al.* 2002).

Akt impacts an array of cellular processes, including cell survival (Chen *et al.* 2001, Vivanco & Sawyers 2002, Yang *et al.* 2003, Downward 2004, Brazil *et al.* 2004) and influences cell survival, in part, through phosphorylation of the proapoptotic protein, BAD (Datta *et al.* 1997, 1999, 2002, del Peso *et al.* 1997, Downward 1999, Bergmann 2002, Jiang *et al.* 2003). Phosphorylation of BAD prevents its association with Bcl-2 or Bcl-X_L, leaving these proteins free to exert their antiapoptotic function (Yang *et al.* 1995, Zha *et al.* 1996). In our experimentation, maternal dexamethasone treatment diminished junctional zone placental Akt activation, which was associated with decreased BAD phosphorylation and increased PARP cleavage, an indicator of apoptosis. These findings are consistent with earlier experimentation showing maternal dexamethasone

increases apoptosis, as measured by TUNEL activity, within the junctional zone of the rat placenta (Waddell *et al.* 2000).

In summary, maternal dexamethasone-induced IUGR is associated with placental insufficiency, dysregulation of placental hormone production, and inhibition of placental IGF-II and Akt signaling. Based on the literature presented above, it is reasonable to presume that some or all of these events may be linked; however, establishing causal relationships will require additional experimentation.

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References

- Ain R, Canham LN & Soares MJ 2003 Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Developmental Biology* **260** 176–190.
- Ain R, Trinh M-L & Soares MJ 2004 Interleukin-11 signaling is required for the differentiation of natural killer cells at the maternal-fetal interface. *Developmental Dynamics* **231** 700–708.
- Barker DJP, Martyn CN, Osmond C, Hales CN & Fall CHD 1993 Growth in utero and serum cholesterol concentrations in adult life. *British Medical Journal* **307** 1524–1527.
- Benediktsson R, Lindsay RS, Noble J, Seckl JR & Edwards CR 1993 Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* **341** 339–341.
- Bergmann A 2002 Survival signaling goes BAD. *Developmental Cell* **3** 607–608.
- Brazil DP, Yang ZZ & Hemmings BA 2004 Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends in Biochemical Sciences* **29** 233–242.
- Chan TO, Rittenhouse SE & Tsichlis PN 1999 AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annual Review of Biochemistry* **68** 965–1014.
- Chen WS, Xu P-Z, Gottlob K, Chen M-L, Sokol K, Shyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T & Hay N 2001 Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene. *Genes and Development* **15** 2203–2208.
- Cheng SL, Zhang SF, Mohan S, Lecanda F, Fausto A, Hunt AH, Canalis E & Aviolo LV 1998 Regulation of insulin-like growth factors I and II and their binding proteins in human bone marrow stromal cells by dexamethasone. *Journal of Cellular Biochemistry* **71** 449–458.
- Cho H, Thorvaldsen JL, Chu Q, Feng F & Birnbaum MJ 2001 Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *Journal of Biological Chemistry* **276** 38349–38352.
- Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C & Reik W 2002 Placenta-specific IGF-II is a major modulator of placental and fetal growth. *Nature* **417** 945–948.

- Dai G, Lu L, Tang S, Peal MJ & Soares MJ 2002 The prolactin family mini-array: a tool for evaluating uteroplacental/trophoblast endocrine phenotype. *Reproduction* **124** 755–765.
- Datta SR, Dudek H, Tao X, Masters S, Gotoh H, Fu Y & Greenberg ME 1997 Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91** 231–241.
- Datta SR, Brunet A & Greenberg ME 1999 Cellular survival: a play in three acts. *Genes and Development* **13** 2905–2927.
- Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ & Greenberg ME 2002 Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Developmental Cell* **3** 631–643.
- DeChiara TM, Efstratiadis A & Robertson EJ 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345** 78–80.
- Downward J 1999 How BAD phosphorylation is good for survival. *Nature Cell Biology* **1** E33–E35.
- Downward J 2004 PI 3-kinase, Akt and cell survival. *Seminars in Cell and Developmental Biology* **15** 177–182.
- Faria TN, Deb S, Kwok SCM, Talamantes F & Soares MJ 1990 Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta. *Developmental Biology* **141** 279–291.
- Gluckman PD 2001 Nutrition, glucocorticoids, birth size and adult disease. *Endocrinology* **142** 1689–1691.
- Jiang K, Zhong B, Ritchey C, Gilvary DL, Hong-Geller E, Wei S & Djeu JY 2003 Regulation of Akt-dependent cell survival by Syk and Rac. *Blood* **101** 236–244.
- Kamei T, Jones SR, Chapman BM, McGonigle KL, Dai G & Soares MJ 2002 The phosphatidylinositol 3-kinase signaling pathway modulates the endocrine differentiation of trophoblast cells. *Molecular Endocrinology* **16** 1469–1481.
- Keppel G 1973 Design and analysis. Englewood Cliffs, NJ: Prentice Hall.
- Knipp GT, Audus KL & Soares MJ 1999 Nutrient transport across the placenta. *Advances in Drug Delivery Reviews* **38** 41–58.
- Kulik G & Weber MJ 1998 Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. *Molecular and Cellular Biology* **18** 6711–6718.
- Kurz H, Zechner U, Orth A & Fundele R 1999 Lack of correlation between placenta and offspring size in mouse interspecific crosses. *Anatomy and Embryology* **200** 335–343.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG & Earnshaw WC 1994 Cleavage of poly(Adp-ribose) polymerase by a proteinase with properties like ice. *Nature* **371** 346–347.
- Levinovitz A & Norstedt G 1989 Developmental and steroid hormonal regulation of insulin-like growth factor expression. *Molecular Endocrinology* **3** 797–804.
- Levitt NS, Lindsay RS, Holmes MC & Seckl JR 1996 Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology* **64** 412–418.
- Liggins GC & Howie RN 1972 A controlled trial of antepartum glucocorticoids treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics* **50** 515–525.
- Lopez MF, Dikkes P, Zurawski D & Villa-Komaroff L 1996 Insulin-like growth factor II affects the appearance and glycogen content of glycogen cells in the murine placenta. *Endocrinology* **137** 2100–2108.
- McDonald TJ, Franko KL, Brown JM, Jenkins SL, Nathanielsz PW & Nijland MJ 2003 Betamethasone in the last week of pregnancy causes fetal growth retardation but not adult hypertension in rats. *Journal of the Society for Gynecologic Investigation* **10** 469–473.
- Moorehead RA, Fata JE, Johnson MB & Khokha R 2001 Inhibition of mammary epithelial apoptosis and sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice. *Cell Death Differentiation* **8** 16–29.
- Nakae J, Kido Y & Accili D 2001 Distinct and overlapping functions of insulin and IGF-I receptors. *Endocrine Reviews* **22** 818–835.
- Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R & Shiota K 2004 The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. *Genes and Cells* **9** 253–260.
- del Peso L, Gonzalez-Garcia M, Page C, Herrera R & Nunez G 1997 Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278** 687–689.
- Price WA, Rong L, Stiles AD & D'Ercole AJ 1992 Changes in IGF-I and -II, IGF binding protein, and IGF receptor transcript abundance after uterine artery ligation. *Pediatric Research* **32** 291–295.
- Richards JS 2001 New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Molecular Endocrinology* **15** 209–218.
- Rogers JF & Dawson WD 1970 Foetal and placental size in a *Peromyscus* species cross. *Journal of Reproduction and Fertility* **21** 255–262.
- Seckl JR 1994 Glucocorticoids and small babies. *Quarterly Journal of Medicine* **87** 259–262.
- Schutt S, Florl AR, Shi W, Hemberger M, Orth A, Otto S, Schulz WA & Fundele RH 2003 DNA methylation in placentas of interspecies mouse hybrids. *Genetics* **165** 223–228.
- Shi W, Lefebvre L, Yu Y, Otto S, Krella A, Orth A & Fundele R 2004 Loss-of-imprinting of Peg1 in mouse interspecies hybrids is correlated with altered growth. *Genesis* **39** 65–72.
- Singh U, Fohn LE, Wakayama T, Ohgane J, Steinhoff C, Lipkowitz B, Schulz R, Orth A, Ropers HH, Behringer RR, Tanaka S, Shiota K, Yanagimachi R, Nuber UA & Fundele R 2004 Different molecular mechanisms underlie placental overgrowth phenotypes caused by interspecies hybridization, cloning, and Esx1 mutation. *Developmental Dynamics* **230** 149–164.
- Smink JJ, Koedam JA, Koster JG & van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *Journal of Endocrinology* **174** 343–352.
- Soares MJ 2004 The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reproductive Biology and Endocrinology* **2** 51 [<http://www.rbj.com/content/2/1/51>]
- Soares MJ & Linzer DIH 2001 Rodent prolactin family and pregnancy. In *Prolactin*, pp 139–167. Ed ND Horseman. Norwell, MA: Kluwer Academic Publishers.
- Soares MJ, Chapman BM, Rasmussen CA, Dai G, Kamei T & Orwig KE 1996 Differentiation of trophoblast endocrine cells. *Placenta* **17** 277–289.
- Sugden MC & Langdown ML 2001 Possible involvement of PKC isoforms in signaling placental apoptosis in intrauterine growth retardation. *Molecular and Cellular Endocrinology* **185** 119–126.
- Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R & Shiota K 2001 Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biology of Reproduction* **65** 1813–1821.
- Trainer PJ 2002 Corticosteroids and pregnancy. *Seminars in Reproductive Medicine* **20** 375–380.
- Vivanco I & Sawyers CL 2002 The phosphatidylinositol 3-kinase-AKT pathway in human cancer. *Nature Reviews Cancer* **2** 489–501.
- Vrana PB, Matteson PG, Schmidt JV, Ingram RS, Joyce A, Prince KL, Dewey MJ & Tilghman SM 2001 Genomic imprinting of a placental lactogen gene in *Peromyscus*. *Development, Genes & Evolution* **211** 523–532.
- Waddell BJ, Hishesh S, Dharmarajan AM & Burton PJ 2000 Apoptosis in rat placenta is zone-dependent and stimulated by glucocorticoids. *Biology of Reproduction* **63** 1913–1917.

- Yang E, Zha J, Jockel J, Boise LH, Thompson CB & Korsmeyer SJ 1995 Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80** 285–291.
- Yang Z-Z, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E & Hemmings BA 2003 Protein kinase Ba/Akt1 regulates placental development and fetal growth. *Journal of Biological Chemistry* **278** 32124–32131.
- Zechner U, Reule M, Orth A, Bonhomme F, Strack B, Guenet, Hameister H & Fundele R 1996 An X-chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrid. *Nature Genetics* **12** 398–403.
- Zechner U, Reule M, Burgoyne PS, Schubert A, Orth A, Hameister H & Fundele R 1997 Paternal transmission of X-linked placental dysplasia in mouse interspecific hybrids. *Genetics* **146** 1399–1405.
- Zechner U, Hemberger M, Constancia M, Orth A, Dragatsis I, Luttgies A, Hameister H & Fundele R 2002 Proliferation and growth factor expression in abnormally enlarged placentas of mouse interspecific hybrids. *Developmental Dynamics* **224** 125–134.
- Zha J, Harada H, Yang E, Jockel J & Korsmeyer SJ 1996 Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14–13–3 not BCL-X(L). *Cell* **87** 619–628.
- Zhou J & Bondy C 1992 Insulin-like growth factor-II and its binding proteins in placental development. *Endocrinology* **131** 1230–1240.

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