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**DEXAMETHASONE TREATMENT OF PREGNANT F0 MICE LEADS TO PARENT
OF ORIGIN-SPECIFIC CHANGES IN PLACENTAL FUNCTION OF THE F2
GENERATION**

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Running head: F0 dexamethasone treatment affects F2 placental function

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36 **ABSTRACT**

37 Dexamethasone (dex) treatment of F0 pregnant rodents alters F1 placental function and adult
38 cardiometabolic phenotype. The adult phenotype is transmitted to the F2 generation without
39 further intervention but whether F2 placental function is altered by F0 dex treatment remains
40 unknown. In this study, F0 mice were untreated or received dex ($0.2 \mu\text{g g}^{-1}\text{day}^{-1}$ s.c.) over
41 days (D) 11-15 or D14-18 of pregnancy (term D21). Depending on the period of F0 dex
42 treatment, F1 offspring were lighter at birth or grew more slowly until weaning ($P<0.05$).
43 Glucose tolerance (1g kg^{-1} ip) of adult F1 males was abnormal. Mating F1 males dex exposed
44 prenatally with untreated females had no effect on F2 placental function on D19 of
45 pregnancy. Contrastingly, when F1 females were mated with untreated males, F2 placental
46 clearance of the amino acid analogue, ^{14}C -methylaminoisobutyric acid, was increased by
47 75% at D19 specifically in dams prenatally dex exposed at D14-18 ($P<0.05$). Maternal
48 plasma corticosterone was also increased but F2 placental *Slc38a4* expression was decreased
49 in these dams ($P<0.05$). F0 dex treatment had no effect on F2 fetal or placental weights
50 irrespective of lineage. Effects of F0 dex exposure are, therefore, transmitted
51 intergenerationally to the F2 placenta via the maternal but not paternal line.

52

53 INTRODUCTION

54 Antenatal treatment with the synthetic glucocorticoid, dexamethasone (dex) is used clinically
55 to induce fetal maturation in women threatened with preterm delivery. Despite the benefits to
56 neonatal survival, antenatal dex exposure is associated with reduced size at birth, especially
57 when glucocorticoid dosage is repeated or inappropriately timed (Khan *et al.* 2011). In turn,
58 low birth weight leads to increased cardiometabolic disease risk in later life (Barker 1994).
59 When dex is given to (F0) pregnant rodents, fetal and placental growth is restricted (Smith
60 2003; Ain 2005; Baisden *et al.* 2007; Cuffe *et al.* 2011; Xu *et al.* 2011; Vaughan *et al.* 2013)
61 and the offspring (F1) develop high blood pressure, hyperglycaemia and insulin resistance
62 (Benediktsson *et al.* 1993; Nyirenda *et al.* 1998; Sugden *et al.* 2001; O'Sullivan *et al.* 2013).
63 More recent animal studies have demonstrated that the phenotype programmed in the F1
64 offspring by prenatal glucocorticoid overexposure can be transmitted to *their* offspring (F2),
65 via either the mother or father, in the absence of further treatment (Drake *et al.* 2005; Crudo
66 *et al.* 2012; Iqbal *et al.* 2012; Long *et al.* 2013a; Long *et al.* 2013b; Radford *et al.* 2014).
67 Depending on whether it is transmitted through the male or female line, passage of F1
68 phenotypic traits to the F2 and subsequent generations may be a consequence of germ-line
69 independent changes in maternal intrauterine environment or stable epigenetic changes to the
70 chromatin inherited at fertilisation (Radford and Ferguson-Smith 2011). As the placenta is
71 both zygote-derived and in direct contact with the maternal environment, changes in placental
72 phenotype may mediate the physiological effects of grandparental environmental insults on
73 the F2 fetus.

74

75 In the F1 generation, placental structure is altered as a direct consequence of dex treatment of
76 the F0 pregnant mother (Hahn *et al.* 1999; Hewitt *et al.* 2006; Cuffe *et al.* 2011; O'Connell *et al.*
77 *et al.* 2013; Vaughan *et al.* 2013). Often, dex causes a reduction in the proportion of the
78 labyrinthine zone (Lz) responsible for materno-fetal exchange, along with changes in the size
79 and density of Lz blood vessels (Hewitt *et al.* 2006; O'Connell *et al.* 2011; Vaughan *et al.*
80 2013). Amino acid transport by the F1 placenta is also altered near term in pregnant mice
81 given dex orally or by injection (Audette *et al.* 2011; Vaughan *et al.* 2013). In the F2 progeny
82 of F1 rats exposed to dex *in utero*, there is altered placental expression of genes including the
83 amino acid transporter genes, along with parent of origin-specific changes in fetoplacental
84 growth (Drake *et al.* 2011). Similarly, F1 prenatal exposure to nutritional manipulations that
85 are known to raise endogenous glucocorticoid concentration, alters expression of nutrient

86 transporters in the F2 mouse placenta (Isganaitis *et al.* 2011; Radford *et al.* 2012). However,
87 whether there are structural and functional changes in the actual nutrient transport capacity of
88 the F2 placenta as a result of dex treatment of the pregnant grandmother remains unknown.

89

90 Therefore, this study quantified F2 placental clearance of the non-metabolisable amino acid
91 analogue, 14C-methylaminoisobutyric acid (MeAIB), and the volume of the placental Lz and
92 junctional zone (Jz) in pregnant mice that were either exposed prenatally to dex and mated to
93 untreated males or were untreated themselves and mated with males exposed to dex *in utero*.
94 The System A/SNAT family of transporters that transfer MeAIB across the placenta are
95 known to be essential for normal fetal growth (Cramer *et al.* 2002). Dex was administered to
96 F0 dams at a dose similar to that given to pregnant women threatened with preterm delivery
97 (Brownfoot *et al.* 2008). When given to pregnant animals in previous studies, this dex dosage
98 is known to program abnormal cardiometabolic phenotype in the first-generation offspring
99 (Benediktsson *et al.* 1993; Dodic *et al.* 1998; Nyirenda *et al.* 1998; Woods and Weeks 2005;
100 de Vries *et al.* 2007). F0 females were injected with dex either over the period of maximal
101 placental or fetal growth (Coan *et al.* 2004), whilst controls were untreated in order to remove
102 the known stress of injection *per se* (Meijer *et al.* 2006; Drude *et al.* 2011; Vaughan *et al.*
103 2013). The study was designed to test the hypothesis that F2 placental phenotype would
104 depend on the timing of F0 dex treatment and on whether treatment was of the maternal or
105 paternal grandmother.

106 **Methods**

107 **Animals**

108 All procedures were conducted under the Animals (Scientific Procedures) Act 1986 by UK
109 Home Office license holders. C57BL6/J mice (Harlan, UK) were housed in 12hr:12hr
110 dark:light conditions with *ad libitum* access to food and water throughout. Sixteen female
111 mice were mated with stud males and the day of copulatory plug detection designated as day
112 (D) 1 of pregnancy. Pregnant females (F0) were given a subcutaneous injection of dex (0.2
113 $\mu\text{g g}^{-1} \text{ day}^{-1}$ in 0.2 ml sterile saline vehicle) on the morning of each of five days, from D11 to
114 D15 ($n=6$) or from D14 to D18 ($n=5$). Control females ($n=5$) were left untreated. Females
115 were allowed to litter normally at term and to suckle their own offspring. Neonates (F1) were
116 weighed and sexed, and litter size reduced to four, balanced by sex where possible. Pups were
117 weaned at three weeks of age, ear-notched for identification then housed in groups of the
118 same sex but from mixed litters and maternal treatment groups. Body weight was recorded
119 weekly before and after weaning. Upon reaching maturity, 6-8 week old *F1* females (control
120 $n=7$; D11-15 dex $n=6$, D14-18 dex $n=6$) were mated with stud males. Twelve to fourteen
121 week old *F1* male mice (control $n=7$, D11-15 dex $n=9$, D14-18 dex $n=14$) were mated with
122 virgin untreated females, which received no further treatment during pregnancy (control
123 $n=10$, D11-15 dex $n=13$, D14-18 dex $n=18$). Between 1 and 3 pregnancies were successfully
124 sired by each *F1* male.

125

126 **Glucose tolerance test**

127 After mating, a glucose tolerance test (1 g kg^{-1} , 10% w/v in sterile saline *i.p.*) was carried out
128 on the F1 males after an overnight fast at 14 weeks of age. Glucose concentrations were
129 measured before and at 15, 30, 45, 60, 90 and 120 minutes after glucose injection in a small
130 quantity of blood taken from the tail vein (OneTouch glucometer, Orthoclinical Diagnostics).
131 Two weeks later, males were anaesthetized (fentanyl-fluanisone:midazolam:water, 1:1:2,
132 $10\mu\text{l g}^{-1}$ body weight) and a blood sample collected for corticosterone measurement (ELISA,
133 IBL International, Hamburg, Germany) before euthanasia by cervical dislocation for tissue
134 collection.

135

136 **F2 placental phenotype**

137 Pregnant F1 females, and untreated females mated with F1 males were anaesthetized on D19
138 of pregnancy and unidirectional materno-fetal clearance of ¹⁴C-methylaminoisobutyric acid
139 (100µl, NEN NEC-671, specific activity 1.86 GBq mmol⁻¹) determined as described
140 previously (Vaughan *et al.* 2012). A cardiac blood sample was taken up to 4 minutes after
141 injection of the tracer and the mother was killed by cervical dislocation. Plasma was
142 separated by centrifugation for liquid scintillation counting and corticosterone measurement.
143 The gravid uterus was removed and individual fetuses and placentae dissected and weighed.
144 Fetuses were lysed (5ml Biosol, National Diagnostics, UK at 55°C) for liquid scintillation
145 counting. The placenta closest in weight to the mean of each litter was halved and fixed in
146 formaldehyde (4% in 0.1M HEPES) for histological processing and stereological
147 determination of volumes of the Lz and Jz, responsible for the transport and endocrine
148 functions of the placenta (Vaughan *et al.* 2012). The placenta second closest to the mean was
149 snap frozen in liquid nitrogen for later gene expression analysis. Expression of System A
150 amino acid transporter isoforms, *Slc38a1*, *Slc38a2* and *Slc38a4*, was determined relative to
151 *Hprt1* and *Gapdh* using quantitative RT-PCR and the $\Delta\Delta C_t$ method (Vaughan *et al.* 2012).

152

153 **Statistics**

154 All data are mean \pm SEM. F1 glucose tolerance was assessed by repeated-measures two-way
155 ANOVA, with F0 treatment and time from glucose injection as independent factors. The
156 effect of F0 treatment on all other measured variables was determined by one-way ANOVA
157 with Bonferroni post-hoc test. Linear correlations of the measured variables were computed
158 by Pearson's product-moment coefficient.

159

160 **Results**

161 **F1 neonatal weight and postnatal growth**

162 All F0 females littered on D21 of pregnancy. Litter size was similar in all treatment groups
163 (average 6.6 pups, range 4-8 pups); however, there was a significant effect of F0 dex
164 treatment on F1 birth weight (Table 1). Newborn control and D11-15 dex treated pups were
165 similar in weight whereas those exposed to dex from D14 to D18 were 8% lighter (Table 1).
166 Conversely, during suckling, D11-15 dex pups gained weight more slowly (fractional growth
167 rate, $P < 0.05$ for males and females combined) such that by weaning at 3 weeks of age both
168 males and females were lighter than controls and D14-18 dex pups (Table 1). Although both
169 groups of dex offspring grew faster than controls after weaning ($P < 0.05$), D11-15 dex treated
170 animals remained lightest at week 6, when the females were mated (Table 1).

171

172 **F1 male lineage**

173 At 14 weeks of age, body weight was similar in the three groups of F1 males (control $30.3 \pm$
174 1.1 , dex D11-15 30.7 ± 0.7 , dex D14-18 31.0 ± 0.7 g, $P > 0.05$). After glucose administration,
175 the maximum increment in blood glucose concentration from baseline values was
176 significantly less in both dex exposed groups than in the controls (Fig. 1). Moreover, blood
177 glucose concentration remained elevated above baseline for longer in dex males (Fig. 1,
178 $P < 0.05$, one sample t-test of 120 min glucose versus zero), than in the controls ($P > 0.05$).
179 However, neither the area under the curve of blood glucose with time (Fig. 1) nor the basal
180 fasting glucose concentrations differed with F0 treatment (control 6.9 ± 0.4 , dex D11-15 7.4
181 ± 0.4 , dex D14-18 8.2 ± 0.4 mM, $P > 0.05$). There was also no significant effect of F0
182 treatment on F1 plasma corticosterone concentrations at 16 weeks of age (control 233 ± 34 ,
183 dex D11-15 170 ± 15 , dex D14-18 189 ± 9 ng ml⁻¹). Heart weight was significantly greater in
184 D14-18 dex treated males compared to the other two groups, both as a percentage of total
185 body weight and an absolute value (control 142 ± 2 , dex D11-15 144 ± 7 , dex D14-18 $159 \pm$
186 4 mg, $P < 0.05$).

187

188 When F1 adult males were mated with untreated females, neither maternal weight nor F2
189 fetal and placental weights differed between the three groups at D19 of pregnancy (Table 2).
190 Materno-fetal clearance of MeAIB across the F2 placenta and placental expression of the
191 *Slc38a* genes were also similar in control and dex groups (Figure 2A and B). The Lz and Jz
192 volumes of the F2 placentae, determined by stereology, did not differ with paternal prenatal
193 treatment (Table 2). Neither did plasma corticosterone concentrations of the D19 pregnant

194 females differ with paternity of their offspring (control 603 ± 64 ng/ml, D11-15 dex 540 ± 42
195 ng/ml, D14-18 dex 687 ± 93 ng ml⁻¹, P>0.05).

196

197 **F1 female lineage**

198 Body weight of the F1 females at D19 of their pregnancy differed with F0 treatment: those
199 exposed prenatally to dex from D11 to 15 were lightest whereas the controls were heaviest
200 (Table 2). In particular, the weight of the gravid uterus was less in F1 dams exposed to dex
201 prenatally at D11-15 than in the other two groups. However, F2 litter size and fetal and
202 placental weights were unaffected by the treatment of their maternal grandmother (Table 2).
203 Clearance of MeAIB across the F2 placenta was 75% greater in F1 dams exposed prenatally
204 to dex at D14-18, compared to the other two groups (Figure 2C). In contrast, expression of
205 *Slc38a4*, but not *Slc38a1* or *Slc38a2*, was significantly lower in the F2 placentae of the F1
206 mothers dex exposed at D14-18, compared to the control group (Fig 2D). At D19 of
207 pregnancy, plasma corticosterone concentrations were elevated above control values ($644 \pm$
208 48 ng ml⁻¹, n = 5) in F1 dams exposed prenatally to dex at D14-18 (1119 ± 131 ng ml⁻¹, n = 6,
209 P<0.05) but not at D11-15 dams (600 ± 38 ng ml⁻¹, n = 5, P>0.05). The volumes of the Lz
210 and Jz in the F2 placenta were similar in the three groups of F1 dams (Table 2).

211

212 **DISCUSSION**

213 This study is the first to demonstrate altered function of the F2 mouse placenta as a
214 consequence of treating F0 pregnant dams with the synthetic glucocorticoid, dexamethasone.
215 F1 neonates of F0 dams treated with dex close to term were growth restricted at birth but
216 subsequently caught up in weight with controls whereas those exposed to dex earlier in
217 gestation had normal birth weights but grew poorly in the period immediately after birth.
218 Irrespective of the timing of F0 dex treatment, male F1 offspring had abnormal glucose
219 tolerance as adults. However, prenatal dex exposure of the F1 males had no effect on F2 fetal
220 weight or placental phenotype. In contrast, prenatal dex exposure of F1 females led to an
221 increase in F2 placental MeAIB transport that was specific to the grandprogeny of F0 dams
222 treated with dex near to term.

223

224 Previously, dex treatment either from D11 to D15 or from D14 to D18 of mouse pregnancy
225 has been shown to reduce fetal and placental weight at D19 (Vaughan *et al.* 2013). However,
226 in the current study growth restriction was only seen at birth in the F1 neonates prenatally
227 exposed to dex over the later of these two periods of F0 treatment. This indicates that
228 compensatory increases in fetal growth can occur during late gestation in mice but only when
229 there are more than 2 days between the end of the insult and birth. The growth restricted F1
230 neonates exposed to dex from D14-18 did attain normal weight by 3 weeks after birth,
231 consistent with the catch-up growth reported in human infants of low birth weight (Morrison
232 *et al.* 2010). In contrast, growth of the F1 neonates exposed to dex from D11-15 was slow
233 after birth so that they were lighter than the other two groups at weaning and remained so for
234 the rest of the experiment, despite a normal birth weight. This may be a consequence of
235 reduced absorption of nutrients from the neonatal gut, or changes in the composition or
236 volume of milk produced by the dams during lactation (Drozdowski *et al.* 2009). Placental
237 production of lactogenic hormones may have been impaired by dex administration as the dex
238 exposed placenta is known to weigh less at the end of treatment and remain small at D19
239 after treatment from D11-15 (Vaughan *et al.* 2013). Indeed, the higher growth rate seen in
240 both groups of dex exposed F1 offspring relative to the controls after weaning suggests that
241 there is a degree of growth constraint during the period of suckling after dex treatment of
242 their mothers (Vaughan *et al.* 2013). With group-housed animals, food intake of the F1
243 offspring could not be determined in the current study so nothing is known about the
244 potential contribution of glucocorticoid programmed changes in appetite to postnatal growth
245 (Moisiadis and Matthews 2014; Ross and Desai 2014).

246

247 In common with the adult offspring of rats treated with dexamethasone in late pregnancy
248 (Lindsay *et al.* 1996a; Lindsay *et al.* 1996b; Nyirenda *et al.* 1998; Woods and Weeks 2005;
249 Franko *et al.* 2010) adult F1 male mice of F0 dex treated dams had abnormal glucose
250 tolerance in the current study with a glucose increment that was smaller initially but more
251 prolonged than seen in the control animals. Reduced maximum blood glucose increment may
252 suggest improved glucose tolerance and is consistent with the faster post-weaning weight
253 gain of the dex exposed F1 animals. However, increased glucose uptake in the juvenile
254 animals may lead to greater fat deposition and eventually insulin resistance in old age
255 (Nyirenda *et al.* 1998). Alternatively, the smaller rise in blood glucose level in
256 dexamethasone exposed F1 male mice may be due to impaired absorption of the glucose
257 injected intraperitoneally. The increases in heart weight in these F1 males also suggests that
258 there are changes in adult cardiovascular function as a result of prenatal dex exposure, as seen
259 in other species (Woods and Weeks 2005). Maternal dex treatment during pregnancy has also
260 been shown to alter the response of the hypothalamic-pituitary-adrenal (HPA) axis to stress in
261 the adult offspring in rats and other species (Shoener *et al.* 2006; Hauser *et al.* 2009; Iqbal *et al.*
262 *et al.* 2012; Long *et al.* 2013a). However, there was little evidence of programmed changes in
263 adrenocortical secretion in the F1 dex exposed adult males studied here, as plasma
264 corticosterone concentration did not differ with F0 treatment. Other than body weight, no
265 phenotypic data was collected on non-pregnant *F1* female mice in the present study as all
266 were mated in order to create the F2 generation. Metabolic phenotyping of these females, for
267 example by measuring glucose tolerance, either before or during pregnancy may also have
268 confounded the F2 outcomes by introducing additional stresses.

269

270 There were no differences in fetoplacental weight, placental MeAIB clearance or placental
271 structure between F2 litters of control and F1 dex-exposed male mice. Previous studies in rats
272 have shown inheritance of metabolic traits to F2 adults via the male offspring of F0 dams
273 treated with a similar dose of dex in late pregnancy (Drake *et al.* 2005). These changes
274 associated with a small decrease in F2 placental weight at day 20 of pregnancy (Drake *et al.*
275 2011). However, the present data do not support a major role for the placenta in transmitting
276 a dex programmed F1 phenotype to the F2 generation patrilineally in mice. Alternative
277 mechanisms, for example epigenetic changes in imprinted or other genes more influential in
278 post-natal growth and glucose tolerance than placental phenotype, may result in germ-line

279 dependent paternal intergenerational inheritance, as occurs following undernutrition of F0
280 pregnant mice (Radford *et al.* 2014).

281

282 When F1 female offspring were bred with control males, there was a reduction in the weight
283 of the entire gravid uterus specifically in those dams exposed to dex between D11 to D15 of
284 their intrauterine development. As F1 females from this group were lighter than controls at
285 weaning, and tended to remain so at mating, there may have been a greater degree of
286 maternal constraint on expansion of the uterus and its contents during pregnancy, despite no
287 significant differences in weights of the individual F2 fetuses and placentae. F1 dams given
288 dex prenatally from D14 to D18 of gestation had raised plasma corticosterone and greater
289 materno-fetal MeAIB clearance, a measure of F2 placental System A amino acid transport
290 capacity, on D19 of their own pregnancy. In common with our previous findings (Vaughan *et*
291 *al.* 2012; Vaughan *et al.* 2013), increased System A activity was not explained by changes in
292 gene expression of *Slc38a* transporters. Indeed, F2 placental expression of the imprinted
293 *Slc38a4* gene was reduced significantly in D14-18 dams that had the highest F2 placental
294 MeAIB clearance. In contrast, *Slc38a4* expression is increased in the F2 rat placenta after
295 exposure of the F1 mother to dex prenatally in late gestation (Drake *et al.* 2011) but its
296 expression is unaltered in the F2 placenta following F1 prenatal undernutrition in mice
297 (Radford *et al.* 2012). Species differences in the effects of F0 manipulations on F2 placental
298 *Slc38a4* abundance may reflect yet unidentified morphological changes in the placenta or
299 promoter-specific regulation of the various *Slc38a4* transcripts in the present and previous
300 studies (Constancia *et al.* 2005).

301

302 In the present study, the F1 pregnant dams with the greatest placental capacity for System A
303 amino acid transport had the highest maternal corticosterone concentrations. In previous
304 studies, placental MeAIB clearance was *reduced* when corticosterone was given directly to
305 pregnant mice for several days in late gestation (Vaughan *et al.*, 2012). The high
306 corticosterone concentration observed in the pregnant F1 dam exposed prenatally to dex from
307 D14-18 may, therefore, be due to an elevated but transient HPA response to the acute stress
308 of handling and terminal anaesthesia in line with the enhanced HPA responsiveness seen in
309 adult offspring of other species after prenatal exposure to dex (Liu *et al.* 2001; Shoener *et al.*
310 2006). Alternatively, since transplacental transfer of fetal corticosterone can contribute to
311 maternal levels during mouse pregnancy (Barlow *et al.* 1974; Montano *et al.* 1993; Cottrell *et*
312 *al.* 2012), the high maternal corticosterone levels may reflect enhanced HPA activity of the

313 F2 fetuses. Certainly, intergenerational transmission of an altered HPA phenotype is seen in
314 other species after F0 dex treatment (Long *et al.* 2013a; Moisiadis and Matthews 2014). The
315 high corticosterone concentration observed in the pregnant F1 dam exposed prenatally to dex
316 from D14-18 may, therefore, be the consequence rather than the cause of the changes in F2
317 placental function. Programmed adaptations in F1 maternal glucose metabolism may also
318 contribute to the changes in F2 placental function, since prenatal dex exposure is known to
319 alter adult glucose tolerance in F1 females in large animals (Long *et al.* 2012) and in F1
320 males in the present study.

321

322 The mechanism by which F0 dex treatment during mouse pregnancy leads to enhanced amino
323 acid transport of the F2 placenta via the maternal lineage, therefore, remains unclear. It may
324 involve changes in the chromatin, cytoplasm or mitochondria of the oocyte induced during
325 either oogenesis in the F0 dam or folliculogenesis in the F1 mother as seen after
326 periconceptual dietary manipulations of F0 mouse dams (Watkins *et al.* 2008; Igosheva *et*
327 *al.* 2010; Lager *et al.* 2014). Alternatively, increased F1 maternal constraint associated with
328 her poor postnatal growth or programmed changes in the F2 fetus may lead to an increased
329 fetal demand for nutrients, which then results in an increased placental nutrient supply.
330 Enhanced placental amino acid transport has been observed previously in response to fetal
331 demand signals when mismatches between the fetal drive for growth and the placental
332 nutrient supply are induced either nutritionally or genetically (Vaughan *et al.*, 2012). In turn,
333 an altered placental phenotype may influence the maternal metabolic profile by changing the
334 maternal endocrine environment and resource allocation between the mother and her
335 offspring with consequences for subsequent pregnancies and the reproductive performance of
336 the next generations (Fowden and Moore 2012).

337

338 In summary, the effects of F0 dex treatment on F2 placental phenotype depend on the period
339 of F0 treatment and whether the F1 mother or father was overexposed *in utero*. Changes in
340 the F2 placental capacity for amino acid transport were only seen with F0 treatment close to
341 term transmitted via the maternal lineage, even though there were changes in growth in both
342 sexes and metabolic alterations in the F1 males. Taken together, these observations suggest
343 that the placenta can contribute to intergenerational inheritance of phenotypic traits via the
344 female but not the male lineage in mice. However, the epigenetic mechanisms involved in
345 determining the F2 placental phenotype still remain to be established.

346

347 **REFERENCES**

348

349 Ain, R. (2005) Dexamethasone-induced intrauterine growth restriction impacts the placental
350 prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *Journal of*
351 *Endocrinology* **185**(2), 253-263

352

353 Audette, M.C., Challis, J.R., Jones, R.L., Sibley, C.P., and Matthews, S.G. (2011) Antenatal
354 Dexamethasone Treatment in Midgestation Reduces System A-Mediated Transport in the
355 Late-Gestation Murine Placenta. *Endocrinology* **152**, 3561-70

356

357 Baisden, B., Sonne, S., Joshi, R., Ganapathy, V., and Shekhawat, P. (2007) Antenatal
358 Dexamethasone Treatment Leads to Changes in Gene Expression in a Murine Late Placenta.
359 *Placenta* **28**(10), 1082-1090

360

361 Barker, D.J. (1994) 'Mothers, Babies and Disease in Later Life.' (BMJ Publishing Group:
362 London)

363

364 Barlow, S.M., Morrison, P.J., and Sullivan, F.M. (1974) Plasma corticosterone levels during
365 pregnancy in the mouse: the relative contributions of the adrenal glands and foeto-placental
366 units. *J Endocrinol* **60**(3), 473-83

367

368 Benediktsson, R., Lindsay, R.S., Noble, J., Seckl, J.R., and Edwards, C.R. (1993)
369 Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* **341**(8841), 339-
370 41

371

372 Brownfoot, F.C., Crowther, C.A., and Middleton, P. (2008) Different corticosteroids and
373 regimens for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane*
374 *Database Syst Rev*(4), CD006764

375

376 Coan, P.M., Ferguson-Smith, A.C., and Burton, G.J. (2004) Developmental dynamics of the
377 definitive mouse placenta assessed by stereology. *Biol Reprod* **70**(6), 1806-13

378

379 Constancia, M., Angiolini, E., Sandovici, I., Smith, P., Smith, R., Kelsey, G., Dean, W.,
380 Ferguson-Smith, A., Sibley, C.P., Reik, W., and Fowden, A. (2005) Adaptation of nutrient
381 supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental
382 transporter systems. *Proc Natl Acad Sci U S A* **102**(52), 19219-24

383

384 Cottrell, E.C., Holmes, M.C., Livingstone, D.E., Kenyon, C.J., and Seckl, J.R. (2012)
385 Reconciling the nutritional and glucocorticoid hypotheses of fetal programming. *FASEB J*

386

387 Cramer, S., Beveridge, M., Kilberg, M., and Novak, D. (2002) Physiological importance of
388 system A-mediated amino acid transport to rat fetal development. *Am J Physiol Cell Physiol*
389 **282**(1), C153-60

390

391 Crudo, A., Petropoulos, S., Moisiadis, V.G., Iqbal, M., Kostaki, A., Machnes, Z., Szyf, M.,
392 and Matthews, S.G. (2012) Prenatal synthetic glucocorticoid treatment changes DNA
393 methylation states in male organ systems: multigenerational effects. *Endocrinology* **153**(7),
394 3269-83

395

396 Cuffe, J.S., Dickinson, H., Simmons, D.G., and Moritz, K.M. (2011) Sex specific changes in
397 placental growth and MAPK following short term maternal dexamethasone exposure in the
398 mouse. *Placenta* **32**, 981-9
399

400 de Vries, A., Holmes, M.C., Heijnis, A., Seier, J.V., Heerden, J., Louw, J., Wolfe-Coote, S.,
401 Meaney, M.J., Levitt, N.S., and Seckl, J.R. (2007) Prenatal dexamethasone exposure induces
402 changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal
403 axis function. *J Clin Invest* **117**(4), 1058-67
404

405 Dodic, M., May, C.N., Wintour, E.M., and Coghlan, J.P. (1998) An early prenatal exposure
406 to excess glucocorticoid leads to hypertensive offspring in sheep. *Clin Sci (Lond)* **94**(2), 149-
407 55
408

409 Drake, A.J., Liu, L., Kerrigan, D., Meehan, R.R., and Seckl, J.R. (2011) Multigenerational
410 programming in the glucocorticoid programmed rat is associated with generation-specific and
411 parent of origin effects. *Epigenetics* **6**(11), 1334-43
412

413 Drake, A.J., Walker, B.R., and Seckl, J.R. (2005) Intergenerational consequences of fetal
414 programming by in utero exposure to glucocorticoids in rats. *Am J Physiol Regul Integr*
415 *Comp Physiol* **288**(1), R34-8
416

417 Drozdowski, L.A., Iordache, C., Clandinin, M.T., Todd, Z., Gonnet, M., Wild, G., Uwiera,
418 R.R., and Thomson, A.B. (2009) Maternal dexamethasone and GLP-2 have early effects on
419 intestinal sugar transport in their suckling rat offspring. *J Nutr Biochem* **20**(10), 771-82
420

421 Drude, S., Geissler, A., Olfe, J., Starke, A., Domanska, G., Schuett, C., and Kiank-Nussbaum,
422 C. (2011) Side effects of control treatment can conceal experimental data when studying
423 stress responses to injection and psychological stress in mice. *Lab Anim (NY)* **40**(4), 119-28
424

425 Fowden, A.L., and Moore, T. (2012) Maternal-fetal resource allocation: co-operation and
426 conflict. *Placenta* **33 Suppl 2**, e11-5
427

428 Franko, K.L., Forhead, A.J., and Fowden, A.L. (2010) Differential effects of prenatal stress
429 and glucocorticoid administration on postnatal growth and glucose metabolism in rats. *J*
430 *Endocrinol* **204**(3), 319-29
431

432 Hahn, T., Barth, S., Graf, R., Engelmann, M., Beslagic, D., Reul, J.M., Holsboer, F., Dohr,
433 G., and Desoye, G. (1999) Placental glucose transporter expression is regulated by
434 glucocorticoids. *J Clin Endocrinol Metab* **84**(4), 1445-52
435

436 Hauser, J., Feldon, J., and Pryce, C.R. (2009) Direct and dam-mediated effects of prenatal
437 dexamethasone on emotionality, cognition and HPA axis in adult Wistar rats. *Horm Behav*
438 **56**(4), 364-75
439

440 Hewitt, D.P., Mark, P.J., and Waddell, B.J. (2006) Glucocorticoids prevent the normal
441 increase in placental vascular endothelial growth factor expression and placental vascularity
442 during late pregnancy in the rat. *Endocrinology* **147**(12), 5568-74
443

444 Igosheva, N., Abramov, A.Y., Poston, L., Eckert, J.J., Fleming, T.P., Duchon, M.R., and
445 McConnell, J. (2010) Maternal diet-induced obesity alters mitochondrial activity and redox
446 status in mouse oocytes and zygotes. *PLoS ONE* **5**(4), e10074
447

448 Iqbal, M., Moisiadis, V.G., Kostaki, A., and Matthews, S.G. (2012) Transgenerational effects
449 of prenatal synthetic glucocorticoids on hypothalamic-pituitary-adrenal function.
450 *Endocrinology* **153**(7), 3295-307
451

452 Isganaitis, E., Radford, E.J., Lytras, A., Chen, M., Schroeder, J., Sharma, A., Ferguson-
453 Smith, A.C., and Patti, M.-E. (2011) Paternal History of Exposure to Prenatal Undernutrition
454 Alters Placental Expression of Nutrient Transporters and mTor Targets in F2 Offspring:
455 Potential Contribution to Intergenerational Transmission of Diabetes and Obesity Risk.
456 *Journal of Developmental Origins of Health and Disease* **2**, S4
457

458 Khan, A.A., Rodriguez, A., Kaakinen, M., Pouta, A., Hartikainen, A.L., and Jarvelin, M.R.
459 (2011) Does in utero exposure to synthetic glucocorticoids influence birthweight, head
460 circumference and birth length? A systematic review of current evidence in humans. *Paediatr*
461 *Perinat Epidemiol* **25**(1), 20-36
462

463 Lager, S., Samulesson, A.M., Taylor, P.D., Poston, L., Powell, T.L., and Jansson, T. (2014)
464 Diet-induced obesity in mice reduces placental efficiency and inhibits placental mTOR
465 signaling. *Physiol Rep* **2**(2), e00242
466

467 Lindsay, R.S., Lindsay, R.M., Edwards, C.R., and Seckl, J.R. (1996a) Inhibition of 11-beta-
468 hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the
469 offspring. *Hypertension* **27**(6), 1200-4
470

471 Lindsay, R.S., Lindsay, R.M., Waddell, B.J., and Seckl, J.R. (1996b) Prenatal glucocorticoid
472 exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-
473 hydroxysteroid dehydrogenase inhibitor carbenoxolone. *Diabetologia* **39**(11), 1299-305
474

475 Liu, L., Li, A., and Matthews, S.G. (2001) Maternal glucocorticoid treatment programs HPA
476 regulation in adult offspring: sex-specific effects. *Am J Physiol Endocrinol Metab* **280**(5),
477 E729-39
478

479 Long, N.M., Ford, S.P., and Nathanielsz, P.W. (2013a) Multigenerational effects of fetal
480 dexamethasone exposure on the hypothalamic-pituitary-adrenal axis of first- and second-
481 generation female offspring. *Am J Obstet Gynecol* **208**(3), 217 e1-8
482

483 Long, N.M., Shasa, D.R., Ford, S.P., and Nathanielsz, P.W. (2012) Growth and insulin
484 dynamics in two generations of female offspring of mothers receiving a single course of
485 synthetic glucocorticoids. *Am J Obstet Gynecol* **207**(3), 203 e1-8
486

487 Long, N.M., Smith, D.T., Ford, S.P., and Nathanielsz, P.W. (2013b) Elevated glucocorticoids
488 during ovine pregnancy increase appetite and produce glucose dysregulation and adiposity in
489 their granddaughters in response to ad libitum feeding at 1 year of age. *Am J Obstet Gynecol*
490 **209**(4), 353 e1-9
491

492 Meijer, M.K., Spruijt, B.M., van Zutphen, L.F., and Baumans, V. (2006) Effect of restraint
493 and injection methods on heart rate and body temperature in mice. *Lab Anim* **40**(4), 382-91

494
495 Moisiadis, V.G., and Matthews, S.G. (2014) Glucocorticoids and fetal programming part 1:
496 Outcomes. *Nat Rev Endocrinol* **10**(7), 391-402
497
498 Montano, M.M., Wang, M.H., and vom Saal, F.S. (1993) Sex differences in plasma
499 corticosterone in mouse fetuses are mediated by differential placental transport from the
500 mother and eliminated by maternal adrenalectomy or stress. *J Reprod Fertil* **99**(2), 283-90
501
502 Morrison, J.L., Duffield, J.A., Muhlhausler, B.S., Gentili, S., and McMillen, I.C. (2010) Fetal
503 growth restriction, catch-up growth and the early origins of insulin resistance and visceral
504 obesity. *Pediatr Nephrol* **25**(4), 669-77
505
506 Nyirenda, M.J., Lindsay, R.S., Kenyon, C.J., Burchell, A., and Seckl, J.R. (1998)
507 Glucocorticoid exposure in late gestation permanently programs rat hepatic
508 phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes
509 glucose intolerance in adult offspring. *J Clin Invest* **101**(10), 2174-81
510
511 O'Connell, B.A., Moritz, K.M., Roberts, C.T., Walker, D.W., and Dickinson, H. (2011) The
512 Placental Response to Excess Maternal Glucocorticoid Exposure Differs Between the Male
513 and Female Conceptus in Spiny Mice. *Biology of Reproduction*
514
515 O'Connell, B.A., Moritz, K.M., Walker, D.W., and Dickinson, H. (2013) Synthetic
516 glucocorticoid dexamethasone inhibits branching morphogenesis in the spiny mouse placenta.
517 *Biol Reprod* **88**(1), 26
518
519 O'Sullivan, L., Cuffe, J.S., Paravicini, T.M., Campbell, S., Dickinson, H., Singh, R.R.,
520 Gezmish, O., Black, M.J., and Moritz, K.M. (2013) Prenatal exposure to dexamethasone in
521 the mouse alters cardiac growth patterns and increases pulse pressure in aged male offspring.
522 *PLoS ONE* **8**(7), e69149
523
524 Radford, E.J., and Ferguson-Smith, A.C. (2011) Genomic imprinting: Epigenetic control and
525 potential roles in the developmental origins of postnatal health and disease. In 'The Placenta
526 and Human Developmental Programming.' (Eds. G Burton, DJ Barker, A Moffett and K
527 Thornburg). (Cambridge University Press: Cambridge)
528
529 Radford, E.J., Isganaitis, E., Jimenez-Chillaron, J., Schroeder, J., Molla, M., Andrews, S.,
530 Didier, N., Charalambous, M., McEwen, K., Marazzi, G., Sassoon, D., Patti, M.E., and
531 Ferguson-Smith, A.C. (2012) An unbiased assessment of the role of imprinted genes in an
532 intergenerational model of developmental programming. *PLoS Genet* **8**(4), e1002605
533
534 Radford, E.J., Ito, M., Shi, H., Corish, J.A., Yamazawa, K., Isganaitis, E., Seisenberger, S.,
535 Hore, T.A., Reik, W., Erkek, S., Peters, A.H., Patti, M.E., and Ferguson-Smith, A.C. (2014)
536 In utero undernourishment perturbs the adult sperm methylome and intergenerational
537 metabolism. *Science*
538
539 Ross, M.G., and Desai, M. (2014) Developmental programming of appetite/satiety. *Ann Nutr*
540 *Metab* **64 Suppl 1**, 36-44
541

542 Shoener, J.A., Baig, R., and Page, K.C. (2006) Prenatal exposure to dexamethasone alters
543 hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats. *Am J*
544 *Physiol Regul Integr Comp Physiol* **290**(5), R1366-73
545

546 Smith, J.T. (2003) Leptin Distribution and Metabolism in the Pregnant Rat: Transplacental
547 Leptin Passage Increases in Late Gestation but Is Reduced by Excess Glucocorticoids.
548 *Endocrinology* **144**(7), 3024-3030
549

550 Sugden, M.C., Langdown, M.L., Munns, M.J., and Holness, M.J. (2001) Maternal
551 glucocorticoid treatment modulates placental leptin and leptin receptor expression and
552 materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated
553 with hyperleptinaemia in the early-growth-retarded adult offspring. *Eur J Endocrinol* **145**(4),
554 529-39
555

556 Vaughan, O.R., Sferruzzi-Perri, A.N., Coan, P.M., and Fowden, A.L. (2013) Adaptations in
557 placental phenotype depend on route and timing of maternal dexamethasone administration in
558 mice. *Biol Reprod* **89**(4), 80
559

560 Vaughan, O.R., Sferruzzi-Perri, A.N., and Fowden, A.L. (2012) Maternal Corticosterone
561 Regulates Nutrient Allocation to Fetal Growth in Mice. *J Physiol* **590**, 5529-40
562

563 Watkins, A.J., Ursell, E., Panton, R., Papenbrock, T., Hollis, L., Cunningham, C., Wilkins,
564 A., Perry, V.H., Sheth, B., Kwong, W.Y., Eckert, J.J., Wild, A.E., Hanson, M.A., Osmond,
565 C., and Fleming, T.P. (2008) Adaptive responses by mouse early embryos to maternal diet
566 protect fetal growth but predispose to adult onset disease. *Biol Reprod* **78**(2), 299-306
567

568 Woods, L.L., and Weeks, D.A. (2005) Prenatal programming of adult blood pressure: role of
569 maternal corticosteroids. *Am J Physiol Regul Integr Comp Physiol* **289**(4), R955-62
570

571 Xu, D., Chen, M., Pan, X.L., Xia, L.P., and Wang, H. (2011) Dexamethasone induces fetal
572 developmental toxicity through affecting the placental glucocorticoid barrier and depressing
573 fetal adrenal function. *Environ Toxicol Pharmacol* **32**(3), 356-63
574
575
576

TABLES

Table 1 F1 offspring postnatal growth

F1 weight (g)	F0 treatment		
	Control	Dex D11-15	Dex D14-18
Neonates	n=33	n=34	n=36
Birth *	1.30 ± 0.03 ^a	1.27 ± 0.01 ^{ab}	1.19 ± 0.03 ^b
Males	n=7	n=9	n=14
3 weeks*	9.46 ± 0.24 ^a	7.97 ± 0.30 ^b	9.02 ± 0.22 ^a
6 weeks*	21.8 ± 0.4 ^{ab}	20.2 ± 0.7 ^a	22.2 ± 0.5 ^b
FGR 0-3 weeks (g g ⁻¹ week ⁻¹)*	2.09 ± 0.08 ^{ab}	1.79 ± 0.07 ^a	2.19 ± 0.07 ^b
FGR 3-6 weeks (g g ⁻¹ week ⁻¹)*	0.43 ± 0.02	0.50 ± 0.02	0.50 ± 0.02
Females	n=7	n=5	n=5
3 weeks*	9.33 ± 0.32 ^a	7.79 ± 0.47 ^b	9.02 ± 0.22 ^{ab}
6 weeks*	18.8 ± 0.5	17.5 ± 0.2	17.4 ± 0.3
FGR 0-3 weeks (g g ⁻¹ week ⁻¹)	2.08 ± 0.14	1.75 ± 0.12	2.05 ± 0.12
FGR 3-6 weeks (g g ⁻¹ week ⁻¹)	0.33 ± 0.02	0.41 ± 0.02	0.35 ± 0.02

Mean (±SEM) body weight of F1 offspring of F0 dams either untreated (control) or treated with dexamethasone (dex) from days (D) 11-15 or D14-18 of pregnancy. FGR, Fractional growth rate = (final weight - initial weight)/(initial weight × 3 weeks). * indicates significant effect of F0 treatment by one-way ANOVA (P<0.05). Values within rows with different superscripts are significantly different from each other by Bonferroni *post-hoc* test.

Table 2 Biometry of pregnant F1 offspring and F2 conceptuses

Weight	F0 treatment		
	Control	Dex D11-15	Dex D14-18
<i>F1 male x untreated female</i>	n=9 litters	n=13 litters	n=18 litters
Maternal weight (g)	38.6 ± 0.6	36.1 ± 0.8	35.4 ± 1.2
Carcass	25.6 ± 0.3	24.2 ± 0.5	23.5 ± 0.6
Gravid Uterus	13.1 ± 0.7	11.9 ± 0.5	11.8 ± 0.7
F2 fetus (mg)	1207 ± 17	1204 ± 20	1220 ± 15
F2 placenta (mg)	87 ± 6	86 ± 2	84 ± 1
Junctional zone	22 ± 3	21 ± 2	23 ± 3
Labyrinthine zone	47 ± 4	49 ± 1	43 ± 4
Litter size (pups)	7.7 ± 0.5	7.1 ± 0.5	7.0 ± 0.3
<i>F1 female x untreated male</i>	n=5 litters	n=5 litters	n=6 litters
Maternal weight (g)*	37.9 ± 1.0 ^a	33.6 ± 0.9 ^b	35.5 ± 1.0 ^{ab}
Carcass	24.2 ± 0.9	23.4 ± 0.4	22.8 ± 1.4
Gravid Uterus*	13.7 ± 0.5 ^a	10.2 ± 0.9 ^b	12.6 ± 0.6 ^a
F2 fetus (mg)	1125 ± 21	1097 ± 14	1135 ± 38
F2 placenta (mg)	88 ± 5	84 ± 2	85 ± 3
Junctional zone	24 ± 3	26 ± 2	25 ± 4
Labyrinthine zone	54 ± 5	46 ± 3	48 ± 2
Litter size (pups)	7.8 ± 0.6	6.4 ± 0.8	7.5 ± 0.2

Mean (\pm SEM) weights of pregnant mothers, fetuses and placentae on D19 of pregnancy in untreated females mated to F1 male offspring or F1 female offspring mated to untreated males where F1 offspring was either untreated (control) or treated with dexamethasone (DEX) from days (D) 11-15 or D14-18 of the F0 pregnancy * indicates significant effect of F0 treatment by one-way ANOVA ($P < 0.05$). Values within rows with different superscripts are significantly different from each other by Bonferroni *post-hoc* test.

Figure 1

Main figure, mean (\pm SEM) increments in blood glucose concentrations from baseline (0min) values in response to glucose administration at 0 minutes in adult F1 males of F0 dams that were untreated (Controls, open symbols) or treated with dexamethasone (Dex) at days (D) 11-15 (black symbols) or D14-18 (grey symbols). The effects of time and maternal treatment were determined by two-way ANOVA (treatment $P=0.0436$, time $P<0.0001$, interaction $P<0.0001$), different letters a and b indicate different treatment groups at each time point by Bonferroni post test. *Inset*, mean (\pm SEM) area under the curve of blood glucose concentration increment with time from glucose administration in adult F1 males as above. There was no significant effect of maternal treatment by one-way ANOVA. F1 n values: Control $n=7$, dex D11-15 $n=9$, dex D14-18 $n=14$

Figure 2

Mean (\pm SEM) values of materno-fetal MeAIB clearance across F2 placentae (A and C) and of placental *Slc38a* gene expression (B and D) in offspring of F1 fathers (A and B) and F1 mothers (C and D) that were either untreated (controls) or exposed prenatally to dexamethasone (Dex) at days (D) 11-14 or D14-18 of the F0 pregnancy. The effect of F1 prenatal treatment was determined by one-way ANOVA, different letters a and b indicate significantly different values by Bonferroni post-hoc test. (A), Control $n=9$, dex D11-15 $n=13$, dex D14-18 $n=17$. (C), Control $n=4$, dex D11-15 $n=4$, dex D14-18, $n=6$. (B and D) $n=5$ per treatment group.