Placental Nanoparticle-mediated IGF1 Gene Therapy Corrects Fetal Growth Restriction in a Guinea Pig Model

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ABSTRACT

Fetal growth restriction (FGR) caused by placental insufficiency is a major contributor to neonatal morbidity and mortality. There is currently no in utero treatment for placental insufficiency or FGR. The placenta serves as the vital communication, supply, exchange, and defense organ for the developing fetus and offers an excellent opportunity for therapeutic interventions. Here we show efficacy of repeated treatments of trophoblast-specific human *insulin-like 1 growth factor* (*IGF1*) gene therapy delivered in a non-viral, polymer nanoparticle to the placenta for the treatment of FGR. Using the guinea pig maternal nutrient restriction model of FGR, nanoparticle-mediated *IGF1* treatment was delivered to the placenta via ultrasound guidance across the second half of pregnancy, after establishment of FGR. This treatment resulted in correction of fetal weight in MNR animals compared to control, improved fetal physiology and no negative maternal side-effects. Overall, we show for the first time a therapy capable of improving the entire pregnancy environment: maternal, placental, and fetal. This combined with our previous studies using this therapy at both mid pregnancy and in numerous cell and animal models demonstrate the plausibility of this therapy for future human translation to improve health outcomes of neonates and decrease numerous morbidities associated with the developmental origins of disease.

Key Words: Placenta, Fetal Growth Restriction, Placental Insufficiency, Gene Therapy, IGF1

INTRODUCTION

10% of humans worldwide are born too small and too early [1]. This equates to 32 million fetal growth restricted (FGR) newborns including over 2.5 million stillbirths and 15 million preterm deliveries [2-5]. When not victim to stillbirth, FGR neonates are at increased risk of developing complications and morbidities associated with developmental origins of health and disease (DOHaD), which links FGR-associated fetal programming to increased risk of childhood/adult morbidities such as developmental delays/cognitive deficits, cardiovascular disease, and obesity [6, 7]. There is currently no treatment for placental insufficiency or FGR. The only intervention is

early delivery which leads to extended NICU stays. While medicine has made great progress with neonatal care, our ultimate goal is to achieve normal gestational length with upward fetal growth trajectories as long as possible for optimal growth since NICU admissions still have significant negative physical and mental impacts on the infant and parents [8, 9] While there are many intrinsic idiopathic causes of FGR stemming from placenta malformation/function, there are also numerous extrinsic causes. These include maternal stress, maternal comorbidities such as diabetes, nutrient deprivation, or drug/alcohol use. However, the commonality between these intrinsic and extrinsic causes is failure of the placenta (whether due to maldevelopment or malfunction) to provide enough nutrients and oxygen to the fetus for appropriate growth [10-12]. The placenta serves as the vascular, endocrine, and circulatory organ responsible for communication between parent and fetus and offers an excellent opportunity for therapeutic interventions: targeting the origin of the disorder, treating an organ that is discarded after pregnancy, and not directly intervening with the fetus [13]. By correcting fetal growth restriction, the number of stillbirths, premature neonates, and adult comorbidities could be dramatically reduced for an overall healthier human population.

The insulin like growth factor (IGF) signaling axis is a master regulator of placental development and function, regulating cell proliferation/differentiation, vasculature structure, nutrient transport, and hormone production. IGF1 is downregulated in human FGR cases and vital for placental function and fetal growth in transgenic mouse studies [14, 15]. Many other growth factors including vascular endothelial growth factor (VEGF), placental growth factor (PLGF), and epidermal growth factor (EGF) have been investigated for the correction of FGR, but only IGF1 acts to regulate all of these processes [16-18]. Unlike the other growth factors mentioned which are temporally produced in the placenta, IGF1 is produced and necessary throughout the entirety of gestation. Previous studies aiming to increase fetal growth with IGF1 have used IGF1 protein delivered through either the maternal blood supply or amniotic fluid [19, 20]. However, these approaches have failed to impact fetal growth after the time point of current possible clinical diagnosis of fetal growth restriction.

In contrast to previous studies using IGF1 protein, we have developed a non-viral, biodegradable copolymer complexed with a plasmid containing the *IGF1* gene under control of a trophoblast-specific promoter to produce a nanoparticle capable of delivering placenta specific transgene expression [19-27]. Thus far in our nanoparticle therapy studies we have successfully delivered plasmids *ex vivo* and *in vitro* to human syncytiotrophoblast and *in vivo* to mouse, guinea pig, and non-human primate models and shown physiochemical properties, cellular safety, and efficiency in both mother and fetus [23, 24, 28]. We have also shown that administration of this gene therapy to the placenta in a normal pregnancy environment results in homeostatic responses within the placenta maintaining normal fetal growth and no placentomegaly [22, 28]. Previous studies using the guinea pig model of FGR from our lab have focused on short term effects of our nanoparticle-mediated *IGF1* at mid-pregnancy and showed positive improvements to the placenta that could improve fetal growth, so this study employed multiple *IGF1* treatments across later gestation to assess longer term impact on placental function, fetal weight and maternal environment.

In this study we used the well-established, non-invasive maternal nutrient restriction model in the guinea pig, a model of maternal stress which establishes placental insufficiency [29, 30].

Guinea pigs are an excellent model to study placental and fetal development. While mouse models offer genetic tools and many established techniques, the mouse does not offer the best model for placental or fetal development for human translation, as they do not have invasive haemomonochorial placentas or precocial young [31, 32]. Non-human primate studies are currently underway to establish dosing and safety in a pre-clinical model but do not provide a system to model FGR across late pregnancy reflecting the human [28]. Guinea pigs, however, provide a model with similar developmental milestones of fetal and placental development compared to humans including similar hormonal profiles, similar placental structure, and similar fetal and neonatal organ development timelines [31-33]. Here we aimed to identify if repeated delivery o*f* nanoparticle-mediated *IGF1* treatment can correct fetal growth by restoring aberrant placental physiology and signaling and determine impact on the maternal environment. Key aspects of this study include the establishment of the growth restriction prior to treatment and allowing treatment to begin after this diagnosis.

METHODS

Nanoparticle Formation

Nanoparticles were formed by complexing a non-viral $PHPMA_{115}$-b-$PDMEAMA_{115}$ co-polymer with plasmids containing the *hIGF1* gene under the control of a trophoblast-specific promoter, *CYP19A1* (50 µg plasmid in a 200 µL volume) at room temperature. Detailed methods of copolymer synthesis and nanoparticle formation can be found in Wilson et al., 2022 [23].

Animal Husbandry and Nanoparticle Delivery

Animal care and usage was approved by the Institutional Animal Care and Usage Committee at the University of Florida (Protocol #202011236). Female Dunkin-Hartley guinea pigs (Dams) were purchased (Charles River Laboratories, Wilmington, MA) at 500–550 g and housed in an environmentally controlled room (22°C/72°F and 50% humidity) under a 12 h light-dark cycle. Upon arrival, food (LabDiet diet 5025: 27% protein, 13.5% fat, and 60% carbohydrate as % of energy) and water were provided ad libitum. After a 2-week acclimation period, dams were weighed and assigned to either ad libitum diet (termed Control: n = 6) or maternal nutrient restriction (MNR) diet (n=12). Timed matings, pregnancy confirmation ultrasounds, and ultrasound-guided intra-placental nanoparticle treatments were performed as previously described [23]. Briefly, dams were anesthetized, and nanoparticle was delivered to the placenta via ultrasound-guided intra-placental injection, 8 days apart at GD36, 44, and 52 (±3). Only one placenta per litter was injected with either nanoparticle-mediated *IGF1* (n=6 MNR + *IGF1*) or a non-expressing sham nanoparticle (n=12; 6 Control and 6 MNR) (Supplemental Figure S1). In 17 out of 18 litters, the same placenta was injected at each timepoint; in one dam fetal positioning over the placenta following the first treatment prevented subsequent treatments so the next closest placenta was injected for the second and third treatment. Placentas were determined to have either high (an RQ>6) or low (RQ<6) *hIGF1* levels by qPCR analysis post processing which ultimately corresponded with directly injected placentas having high expression and indirectly affected placentas having low expression. Dams were sacrificed at GD60 (±3) via carbon dioxide asphyxiation followed by cardiac puncture and exsanguination. Fetuses (Control: n=8 female and n=11 male, MNR: n=5 female and 11 male, MNR + *IGF1*: n=6 female and 10 male) and the delivered placenta (placenta, sub-placenta, and decidua) were removed from the uterus and weighed. Fetal sex was determined at this time by examination of the gonads. Blood was collected

via cardiac puncture from dams and fetuses at time of sacrifice. Fetal and maternal organs (heart, lung, liver, kidney, spleen, brain (fetus only), were isolated, weighed, and processed for histology (fixed in 4% PFA) or molecular analysis (preserved in RNAlater and snap-frozen).

Quantitative PCR (qPCR)

Approximately 150–200 mg of placenta tissue was lysed and homogenized in RLT-lysis buffer (Qiagen) using a tissue homogenizer. RNA was extracted and DNase treated using the RNeasy Midi Plus Kit (Qiagen) following standard manufacturers protocols. The High-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to convert 1 µg RNA to cDNA, and qPCR reactions were set up and performed on the Quant 3 PCR System (Applied Biosystems) using PowerUp Sybr Green Master Mix (Applied Biosystems), following standard manufacturers protocol. *IGF1* primers were designed for species specificity for both guinea pig (*gpIgf1; Fwd: 5'* – CCTTCTGCTTGCTCGTCC, Rev: 5' -TCTCCAGCCTCCGCAGGTCG) and human *(hIGF1; Fwd: 5'*- CGCTGTGCCTGCTCACCT, Rev: 5' – TCTCCAGCCTCCTTAGATCA) and purchased from Integrated DNA Technologies (IDT). They were then tested using RT-PCR using the Sigma Fast Start PCR mastermix on both gDNA and cDNA for 35 cycles using both human and guinea pig samples to confirm species specificity. Samples were run on a 1.5% agarose gel and imaged using a biorad chemidoc to confirm species specificity before performing qPCR experiments. Commercially available KiCqStart primers (Sigma) were used to measure mRNA expression of guinea pig *Igf2*, *Igf1 Receptor* and *Igf Binding Protein 3*, normalized to *β-actin*, *Gapdh* and *Rsp20*. Relative mRNA expression was calculated using the comparative CT method with the Design and Analysis Software v2.6.0 (Applied Biosystems).

Serum/plasma analysis

Maternal (heparinized, EDTA and serum) and fetal (heparinized) blood samples were collected at time of sacrifice and serum/plasma obtained by centrifugation. Plasma was analyzed for maternal and fetal glucose and lactate using the YSI model 2700 Glucose/Lactate Analyzer following standard protocols. Maternal plasma electrolytes (sodium and potassium) were analyzed using the Roche Electrolyte Analyzer 9180 following standard protocols. Cortisol and progesterone in maternal serum and cortisol in fetal plasma were analyzed using commercially available ELISA kits (Arbor Assays) following manufacturers specifications.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics 29 software. Female and male fetuses were analyzed separately. Distribution assumptions were checked with a Q-Q-Plot. For maternal outcomes, statistical significance was determined using generalized linear modeling with gamma as the distribution and log as the link function. Diet and nanoparticle-mediated *IGF1* treatment were considered main effects, and gestational age and litter size designated covariates. For fetal and placental outcomes, statistical significance was determined using generalized estimating equations with gamma as the distribution and log as the link function. Dams were considered the subject, diet and nanoparticle-mediated *IGF1* treatment treated as main effects and gestational age and litter size as covariates. In male fetuses, high (RQ>6) or low (RQ<6) placental expression of *hIGF1* was also considered as a main effect. Statistical significance was considered at $P \leq 0.05$. For statistically significant results, a Bonferroni post hoc analysis was performed. Results are reported as estimated marginal means ± 95% confidence interval.

RESULTS

IGF signaling is regulated in a sexually dimorphic manner in both Fetal Growth Restriction and following nanoparticle-mediated trophoblast-specific *IGF1* treatment

Repeated nanoparticle-mediated *IGF1* treatments from mid-pregnancy to near term resulted in no maternal health complications, no fetal loss, and no placental hemorrhage. One fetus in each pregnancy was chosen to receive direct nanoparticle injection into its placenta. As fetal sex determination is not possible at time of injection, the sex of the fetuses receiving direct treatment vs indirectly exposed littermates was random. At time of sacrifice fetuses were sexed, and it was determined that, in this study, only the placentas of male fetuses had received direct *IGF1* treatment therefore no direct MNR + *IGF1* treatment category is shown on graphs presenting female fetal data. Female and other male littermates were indirectly exposed to circulating nanoparticle. One dam within the MNR + *IGF1* treatment group was determined to have a singleton pregnancy and was excluded from the study as the physiology of monozygotic pregnancies and polyzygotic pregnancies in guinea pigs differ significantly [34-36].

Repeated nanoparticle treatments with the human *IGF1* gene resulted in the expression *of hIGF1* mRNA within directly injected and indirectly exposed placentas at time of sacrifice (Figure 1A). However, as expected, indirectly exposed placentas had lower *hIGF1* expression than directly injected placentas. Endogenous levels of *gpIgf1* were reduced in MNR placentas in males compared to controls (Figure 1B; $p<0.001$), but this reduction failed to reach significance in the MNR females. No significant difference was seen in endogenous *gpIgf1* expression in MNR + *IGF1* placentas compared to controls in either sex (Figure 1C).

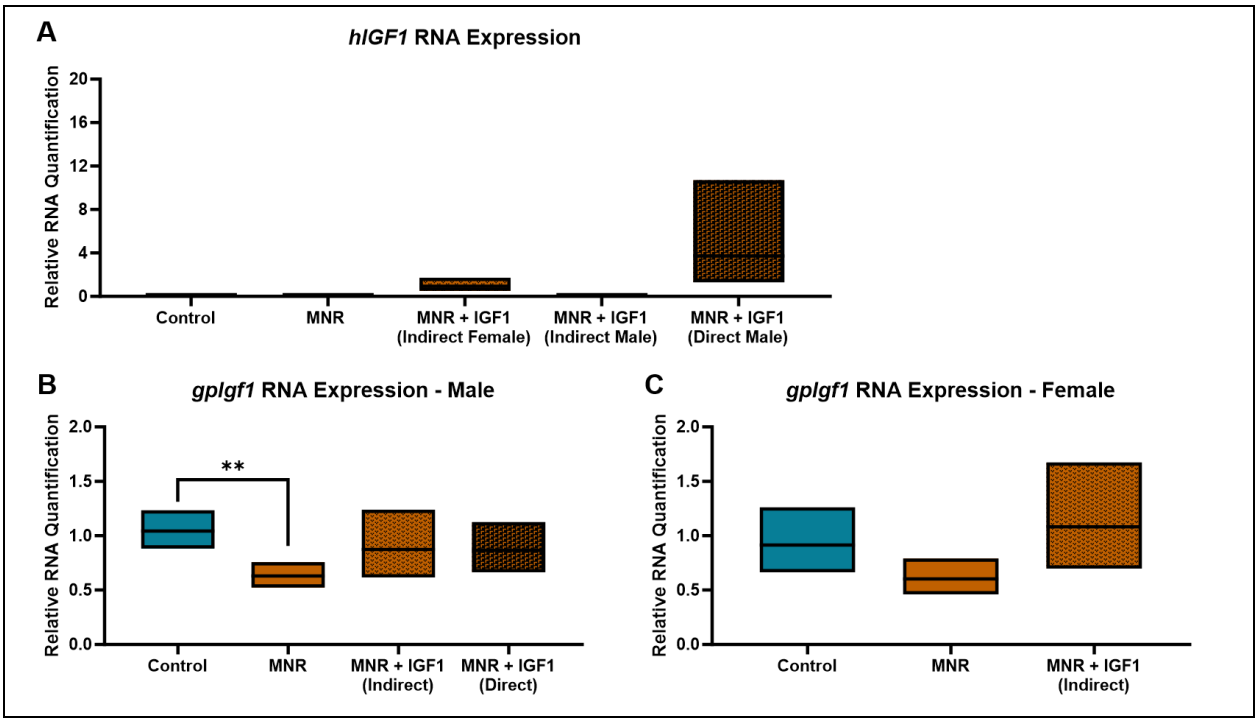

Figure 1. Effects of maternal nutrient restriction (MNR) and repeated nanoparticle-mediated *IGF1* delivery (MNR + *IGF1*) on plasmid-specific human *IGF1* (*hIGF1*) and

endogenous guinea pig *Igf1* (*gpIgf1*). A. In placentas of male fetuses, *hIGF1* levels were increased in the MNR + *IGF1* direct group compared to sham treated Control and MNR groups. In placentas of female fetuses, *hIGF1* levels were increased in the MNR + *IGF1* indirect group compared to sham treated Control and MNR groups. As this data is presence/absence data no statistics are included **B.** Endogenous *gpIgf1* was lower in the sham treated MNR placentas of male fetuses compared to sham treated Control; *gpIgf1* levels in the MNR + *IGF1* groups were comparable to Control. **C.** In placentas of female fetuses, there was no difference in endogenous *gpIgf1* between Control, MNR or MNR + *IGF1*. Control: n = 6 Dams (8 female and 11 male fetuses), MNR: n = 6 Dams (5 female and 11 male fetuses), MNR + *IGF1*: n = 5 Dams (6 female and 10 male fetuses). Data are estimated marginal means ± 95% confidence interval. P values calculated using generalized estimating equations with Bonferroni post hoc analysis. *P≤0.05; **P≤0.01

Sexually dimorphic changes also occurred in the placental expression of other IGF family members including *Igf2, Igf1R,* and *IgfBP3*. *Igf2* expression was not different from control in male MNR placentas (Figure 2A), but significantly increased in female MNR placentas (Figure 2B; P=0.037), indicating a potential compensatory response to maternal dietary restriction/stress by the female but not male placenta. Indirect exposure of males and females to nanoparticle-mediated *IGF1* did not further regulate *Igf2* expression compared to the MNR groups but direct *IGF1* treatment in the male placenta caused increased *Igf2* expression (P=0.056). Indirectly exposed female placentas demonstrated increased *Igf1R* (Figure 2D; P=0.023) and *IgfBP3* (Figure 2F; P=0.02) expression in the MNR + *IGF1* group compared to controls while males showed no change (Figure 2C and 2E, respectively).

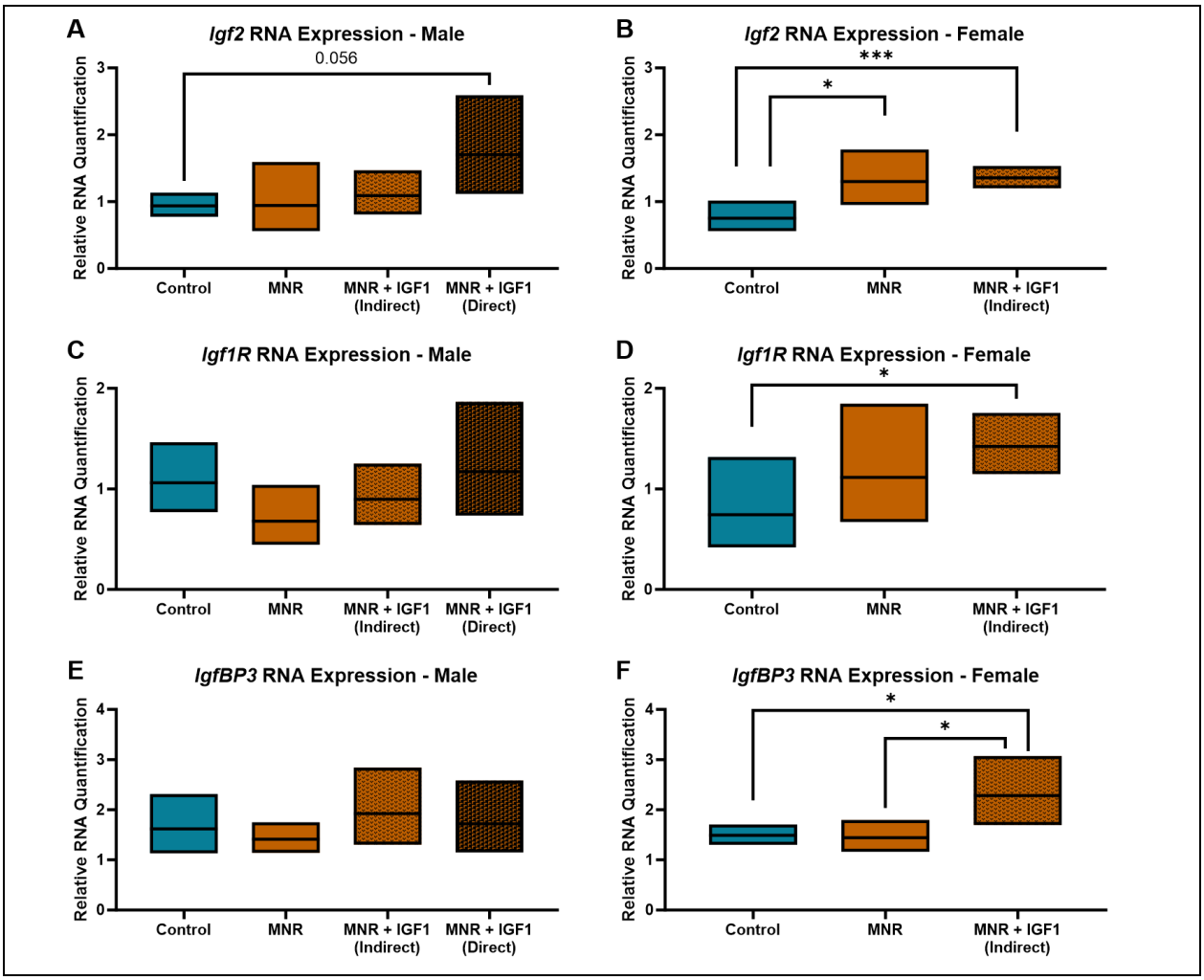

Figure 2. Effect of maternal nutrient restriction (MNR) diet, and repeated *IGF1* delivery on placenta *IGF1* signaling genes: **A.** *Insulin like growth factor 2* (*Igf2*) was unaltered among all male placental groups. **B.** Females in the MNR and MNR + *IGF1* groups had increased *Igf2* placental expression of compared to controls. **C.** *Insulin like growth factor 1 Receptor (Igf1R*) levels were unaltered between placentas of male fetuses. **D.** *Igf1R* expression was increased in MNR + *IGF1* indirect female placentas compared to controls. **E.** *Insulin like growth factor binding partner 3* (*IgfBP3*) was unaltered between placentas of male fetuses. **F.** *IgfBP3* expression was increased in MNR + *IGF1* indirect female placentas compared to control and MNR. Control: n = 6 dams (8 female and 11 male fetuses), MNR: n = 6 dams (5 female and 11 male fetuses), MNR + *IGF1*: n = 5 dams (6 female and 10 male fetuses). Data are estimated marginal means ± 95% confidence interval. P values calculated using generalized estimating equations with Bonferroni post hoc analysis. *P≤0.05; **P≤0.01, ***P≤0.001

Repeated direct nanoparticle-mediated *IGF1* treatment ameliorates fetal growth restriction

Sham treated MNR fetuses of both sexes weighed significantly less than control fetuses at time of sacrifice (Males: Figure 3A; P=0.019, Females: Figure 3B; P=0.003). Fetuses whose placenta received direct *IGF1* treatment (males only directly treated) weighed significantly more than MNR

males (P=0.036) and the same as control males. Weights of male and female fetuses whose placentas were indirectly exposed to *IGF1* treatment demonstrated some recovery towards control weights but were not significantly different from any of the groups.

Males had no changes in delivered placenta weight between any of the groups despite being growth restricted (Figure 3C), while females showed reduced placental weight in the MNR + *IGF1* group compared to controls (Figure 3D. P=0.013). Similarly, males had no changes in delivered placental efficiency across groups (Figure 3E), while females showed a reduction in efficiency with MNR, but this reverted to control levels of efficiency with indirect exposure to nanoparticle-mediated *IGF1* treatment (Figure 3F. MNR P<0.001, MNR + *IGF1* P=0.011).

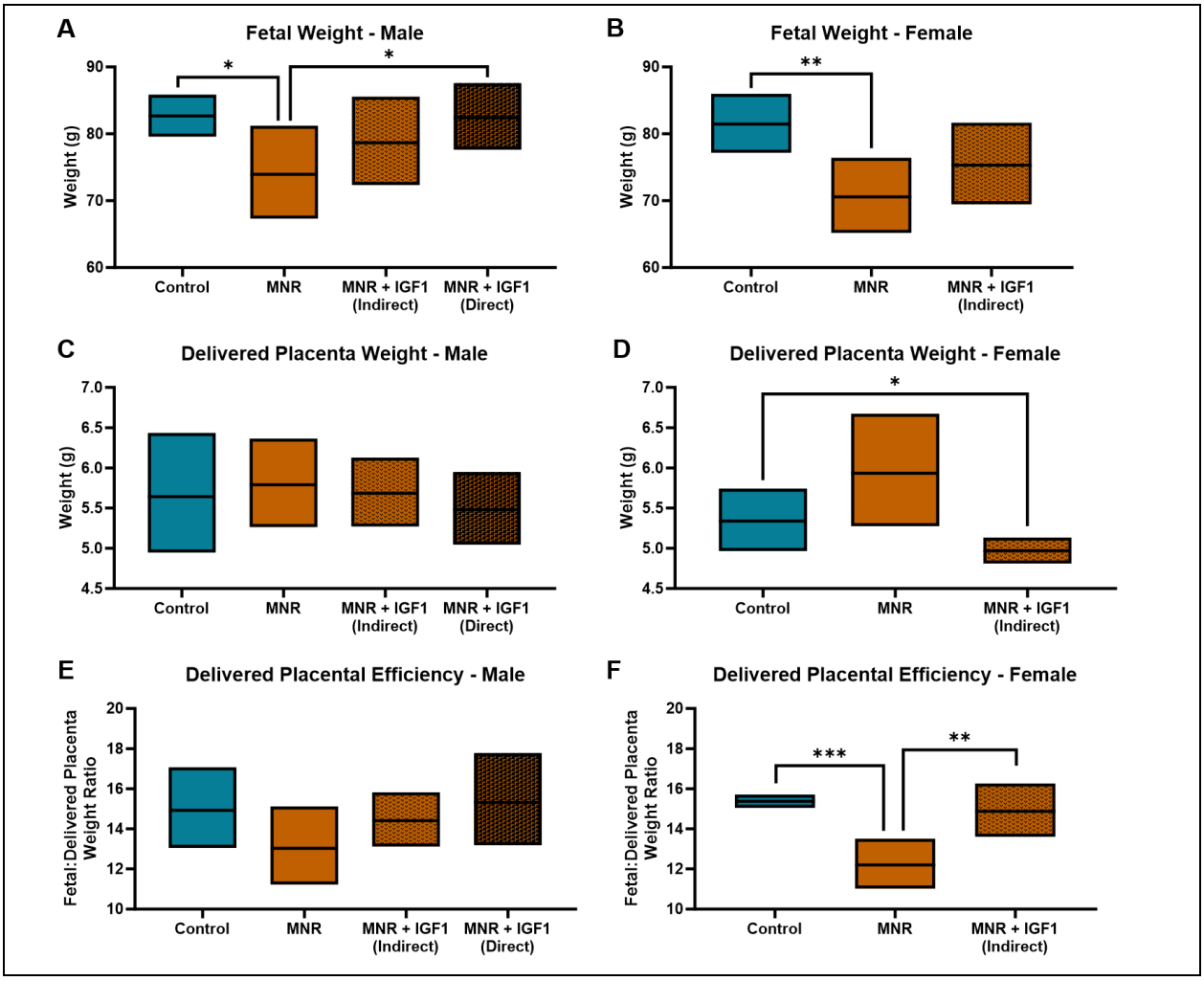

Figure 3. Effect of maternal nutrient restriction (MNR) diet, and repeated *IGF1* delivery on fetal and placental weight A & B. MNR fetuses weight significantly less than controls in both sexes. MNR + *IGF1* (direct and indirect) fetuses showed comparable weights to controls in males and females with full correction from MNR with direct *IGF1* treatment in males. **C.** Delivered placenta weight showed no changes among any groups in male fetuses. **D.** Delivered placenta weight decreased in MNR + *IGF1* compared to controls in females. **E.** Delivered placental efficiency showed no changes among males in any group. **F.** Delivered placental efficiency decreased with MNR in females compared to controls but was corrected in the MNR

+ *IGF1* group. Control: n = 6 (8 female and 11 male), MNR: n = 6 (5 female and 11 male), MNR + *IGF1*: n = 5 (6 female and 10 male). Data are estimated marginal means ± 95% confidence interval. P values calculated using generalized estimating equations with Bonferroni post hoc analysis. *P≤0.05; **P≤0.01, ***P≤0.001

Fetal organs were dissected and weighed at time of necropsy. In the MNR group, male and female fetuses displayed brain sparing with significantly higher brain weights as a percentage of fetal weight compared to controls (Table 1). Demonstrating the impact of improved placenta function/efficiency, brain weight as a percentage of fetal weight was comparable to control in the MNR + IGF1 directly treated male fetuses and the MNR + *IGF1* indirectly treated female fetuses. Other, minor sex-specific changes were observed in fetal liver, kidney and lung weights (percentage fetal weight) with MNR and MNR + *IGF1* treatment compared to control as reported in Table 1.

Table 1. Fetal organ weights between control, MNR and nanoparticle-mediated *IGF1* treatment (MNR + *IGF1*) groups

		Control			**MNR**			**MNR + IGF1 (Indirect)**			**MNR + IGF1 (Direct)**		
		Mean	95% Confidence Interval		Mean	95% Confidence Interval		Mean	95% Confidence Interval		Mean	95% Confidence Interval	
Males	Heart (% Fetal Weight)	0.57	0.50	0.66	0.59	0.49	0.71	0.67	0.60	0.75	0.60	0.48	0.74
	Lung (% Fetal Weight)	2.17^{A}	2.08	2.26	2.14^{A}	2.06	2.22	2.14^{A}	1.78	2.57	2.33^{B}	2.28	2.39
	Liver (% Fetal Weight)	4.94^{A}	4.73	5.17	5.34^{B}	5.08	5.61	5.76^{B}	5.38	6.16	$5.14^{A,B}$	4.98	5.31
	Kidney (% Fetal Weight)	1.07^{A}	0.88	1.30	0.96^{A}	0.88	1.06	0.97^{A}	0.86	1.09	0.85^{B}	0.80	0.91
	Spleen (% Fetal Weight)	0.16^{A}	0.13	0.19	0.14^{A}	0.13	0.16	0.20^{B}	0.16	0.24	0.16^{A}	0.13	0.20
	Brain (% Fetal Weight)	3.05^{A}	2.88	3.24	3.38^{B}	3.19	3.59	2.72^{C}	2.50	2.97	$3.19^{A,B}$	2.96	3.43
	Brain:Liver Weight Ratio	0.62^{A}	0.58	0.67	0.64^{A}	0.60	0.67	0.47^{B}	0.45	0.49	0.62^{A}	0.58	0.67
Females	Heart (% Fetal Weight)	0.57	0.53	0.60	0.64	0.57	0.71	0.56	0.53	0.60			
	Lung (% Fetal Weight)	2.24	2.18	2.30	2.24	2.04	2.47	2.27	2.13	2.41			
	Liver (% Fetal Weight)	$5.27^{A,B}$	4.84	5.74	5.51^{A}	5.14	5.92	5.10^{B}	4.94	5.27			

	Kidney (% Fetal Weight)	0.91	0.86	0.97	1.03	0.91	1.17	0.93	0.88	0.99	
	Spleen (% Fetal Weight)	0.17	0.16	0.17	0.18	0.17	0.19	0.18	0.17	0.19	
	Brain (% Fetal Weight)	3.03[A]	2.85	3.22	3.43[B]	3.18	3.70	3.30[A, B]	2.92	3.73	
	Brain:Liver Weight Ratio	0.59	0.52	0.66	0.62	0.58	0.67	0.65	0.57	0.74	

Data are estimated marginal mean calculated using Generalized Estimating Equation with a gamma-log link function, accounting for gestational day and litter size. Different letters denote significant differences between the row.

Repeated nanoparticle-mediated *IGF1* treatment effectively corrects aberrant fetal glucose and cortisol levels

Glucose, lactate, and cortisol were measured in fetal blood at time of sacrifice. Male fetuses showed significantly reduced fetal blood glucose with MNR when compared to control (Figure 4A; P=0.001). However, MNR + *IGF1* fetuses, both direct and indirect, showed increased blood glucose when compared to MNR and comparable to control (Figure 4A; MNR + *IGF1* (Indirect) P = 0.019, MNR + *IGF1* (Direct) P = 0.039). Females, however, showed no changes in fetal blood glucose between groups (Figure 4B). In contrast, males showed no significant changes in blood lactate levels (Figure 4C), while females showed an increase blood lactate in the MNR and MNR + *IGF1* groups compared to controls (Figure 4D; MNR P=0.006, MNR + *IGF1* P<0.001). Blood cortisol levels were increased with MNR in both males (Figure 4E; P<0.001) and females (Figure 4F; P<0.001) when compared to control. However, blood cortisol levels were reduced with nanoparticle-mediated *IGF1* treatment as there was no difference between MNR + *IGF1* and control fetuses.

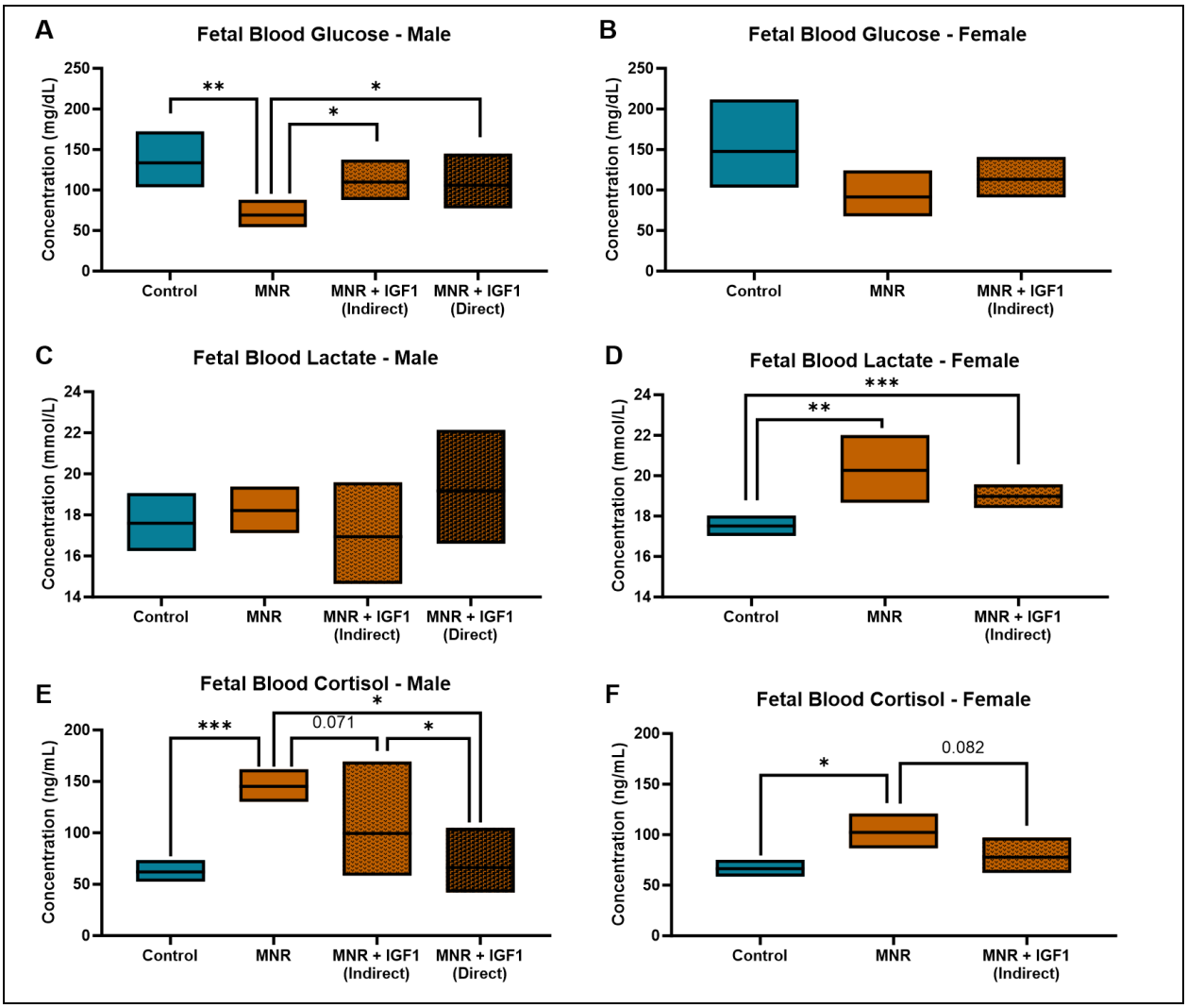

Figure 4. Effect of maternal nutrient restriction (MNR) diet, and repeated IGF1 delivery on fetal blood glucose, lactate, and cortisol A. Male MNR fetuses had lower blood glucose levels compared to controls. MNR + *IGF1* direct and indirect increased blood glucose back to control levels. **B.** There were no changes in female fetal blood glucose levels. **C.** Blood lactate levels were unaltered in male fetuses. **D.** Female fetuses had increased blood lactate with MNR and MNR + *IGF1*. **E & F.** There were increased blood cortisol levels with MNR in both males and females. MNR + *IGF1* male and female fetuses had blood cortisol levels comparable to control. Control: n = 6 dams (8 female and 11 male fetuses), MNR: n = 6 dams (5 female and 11 male fetuses), MNR + *IGF1*: n = 5 dams (6 female and 10 male fetuses). Data are estimated marginal means ± 95% confidence interval. P values calculated using generalized estimating equations with Bonferroni post hoc analysis. *P≤0.05; **P≤0.01, ***P≤0.001

Maternal cortisol levels are elevated with MNR but return to control levels with repeated nanoparticle-mediated *IGF1* placental treatment

Dams had no significant changes in total carcass weight across groups, further indicating the establishment of FGR without using a model of starvation (Supplemental Table S1). Maternal

organs were dissected and weighed. No differences were seen in these weights other than some changes in maternal liver weight shown in supplement (Supplemental Table S1). Maternal blood glucose, lactate, and cortisol levels were measured at time of sacrifice. Maternal blood glucose levels were unaltered between control, MNR and MNR + *IGF1* group, and remained within published physiological levels [37] (Figure 5A). Lactate, the end product of glucose metabolism and 2nd major fuel for fetal oxidative metabolism, was also unaltered between all groups (Figure 5B). Sodium and Potassium levels were also unchanged between all groups (Figure 5C an 5D, respectively). Maternal cortisol levels were significantly increased in the MNR group (P=0.011) but corrected to control levels with treatment in the MNR + *IGF1* group (P=0.013) (Figure 5E). Circulating maternal progesterone levels were unaltered between any of the groups (Figure 5F).

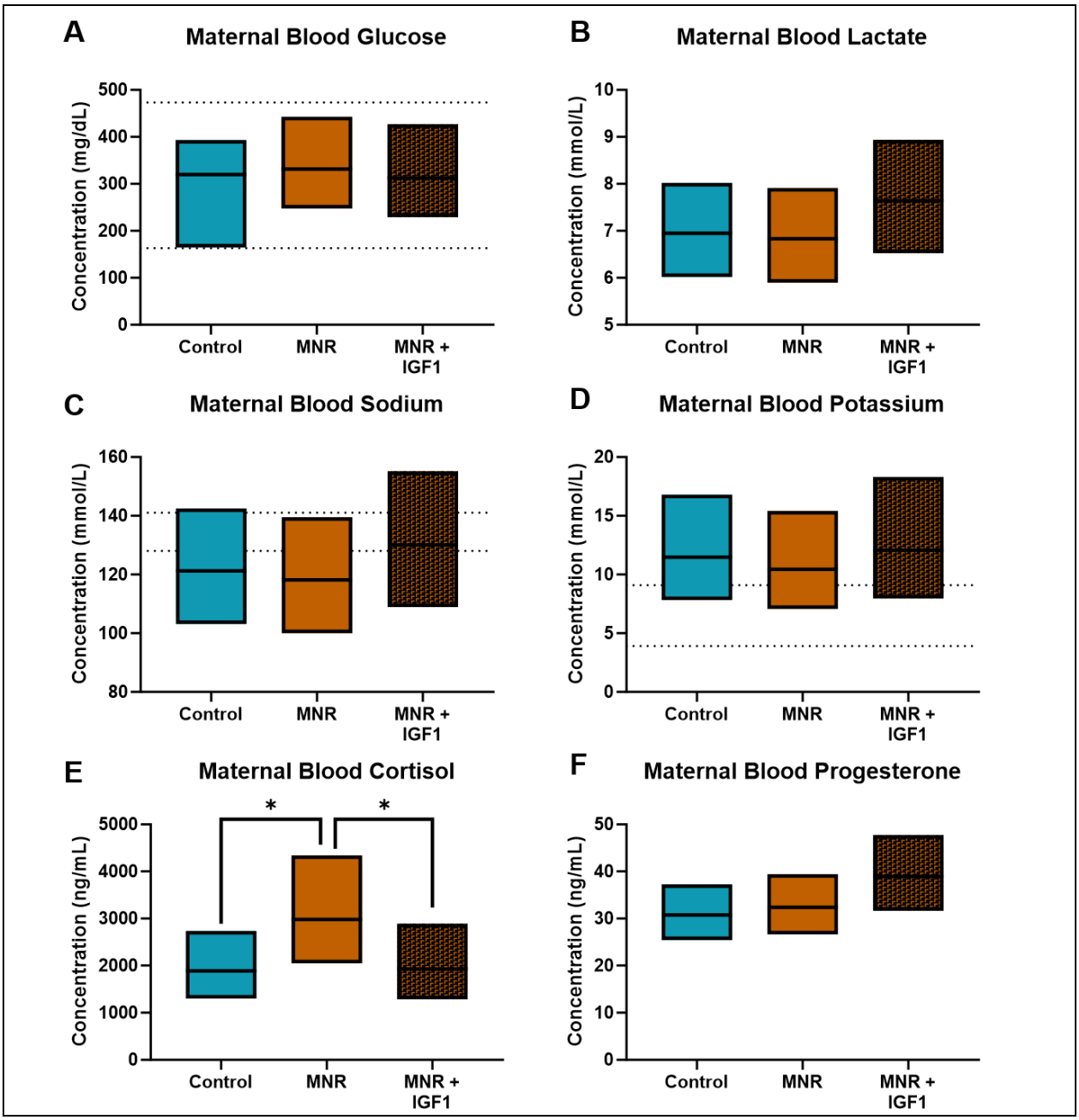

Figure 5. Effect of maternal nutrient restriction (MNR) diet, and repeated nanoparticle-mediated *IGF1* delivery on maternal blood biochemical measures: A. Maternal blood glucose was unaltered among groups **B.** Maternal blood lactate was unaltered among groups **C.** Maternal blood sodium was unaltered among groups **D.** Maternal blood potassium was unaltered among groups **E.** Cortisol was increased with MNR but corrected to control levels with MNR + *IGF1* **F.** Maternal blood progesterone was unaltered among groups. Dotted lines denote normal ranges for each measure in non-pregnant guinea pigs. Control n=6, MNR n=6, MNR + *IGF1* n=5. Data are estimated marginal means ± 95% confidence interval. P values calculated using generalized linear models with Bonferroni post hoc analysis. *P≤0.05; **P≤0.01, ***P≤0.001

DISCUSSION

There are currently no treatments for fetal growth restriction nor placental maldevelopment/dysfunction. To establish a safe and effective treatment for placental insufficiency it is necessary to create a treatment that improves placental development, structure and/or function to increase fetal growth without 1. causing placentamegaly, 2. negatively impacting the maternal environment, or 3. causing negative side-effects to the fetus, or macrosomia in cases of misdiagnosed FGR. Placental *hIGF1* expression following nanoparticle-mediated *IGF1* treatment was associated with comparable fetal weight in male and female MNR + *IGF1* fetuses when compared to control, increased fetal blood glucose, and reduced blood cortisol levels in both fetuses and dams. Overall, this data, in addition to our previous studies on immediate effects of this *IGF1* gene therapy on the placenta, show the potential of this therapy as a treatment for fetal growth restriction that positively impacts placental function, fetal growth, developmental programming, and improves the health of mother and offspring.

It is widely accepted that male fetuses are more susceptible to worse outcomes when affected by in utero disorders and perform more poorly in the NICU than female fetuses and neonates [38, 39]. We previously identified sexually dimorphic responses with both our MNR model and our *IGF1* treatment in fetuses, recapitulating the human scenario [23, 40, 41]. While we had no directly treated female placentas, indirectly treated female placentas showed more significant IGF signaling changes in receptor, binding partner, and *Igf2* mRNA levels than males, highlighting dimorphic responses based on sex. For the first time, we show that female placenta *Igf2* levels increase in response to maternal stress induced placental insufficiency potentially as a means to compensate for poor development and growth whereas male placentas do not. With the expression of *hIGF1* following indirect exposure combined with upregulation of *Igf2* from compensatory response, female placentas upregulate *Igf1R* and *IgfBP3* to likely regulate the bioavailability and response to increased *Igf1.* In contrast, male MNR placentas show no compensatory mechanism and only a trend towards increasing *Igf2* expression following direct *IGF1* treatment.

Both male and female fetuses, irrespective of whether they received direct or indirect *IGF1* treatment, showed improved growth with no statistical differences in fetal weights between controls and the *IGF1* treatment groups; importantly, males receiving direct treatment had rescued weight compared to the MNR group. This shows for the first time our novel treatment's

ability to effectively correct preexisting fetal growth restriction in a model of placental insufficiency. While placentas exposed indirectly show no statistical significance from the MNR group in both sexes, they show an intermediary response with no statistical difference from controls either. The indication of a dose response in fetal weight is clear between direct and indirect treatment and may still represent significant physiological improvement as any weight gain in utero is greatly valued in FGR fetuses. In addition to IGF family member regulation, delivered placenta weights and efficiency also showed sexually dimorphic responses. Whilst we cannot determine the impact of direct *IGF1* treatment to the female placenta from the current study, our data, in addition to historic reporting of females performing better in utero and in NICU under the same conditions as males, indicates the possibility that females may require a lower dosage compared to males for full correction of FGR [38].

In addition to improving placental signaling and fetal growth, repeated nanoparticle-mediated *IGF1* treatment impacted fetal brain growth. Asymmetric growth is a characteristic of 70-80% of all human FGR cases (and the MNR guinea pig model) with disproportionate growth causing increased blood supply to the fetal brain to maintain life and resulting in brain sparing effects [42, 43]. This brain sparing maintains brain growth and leads to an increase in fetal brain weight as a percentage of total body weight compared to average for gestational age (AGA) or symmetrical FGR fetuses [44,45]. We found brain sparing effects in both male and female fetuses in our MNR group, but importantly comparable brain:body ratios in the *IGF1* treatment groups to controls, demonstrating that we are correcting full body weight and asymmetric growth. As a consequence of asymmetrical growth, organs such as the liver, kidneys, and spleen may be deprived of blood flow and growth and development are impaired. However, while some studies show reduction in these organ weights, others show no change in weight, but strong functional deficits [12, 46, 47]. The abnormal growth and development of these organs from FGR are what lead to childhood and adulthood morbidities that increase risks associated diseases such as fatty liver disease, obesity, and cardiovascular disease [6, 7, 32]. Studies identifying the precise malperfusions and functional consequences have been and continue to be investigated for these various organ systems [48-53]. We are the first to show a treatment correcting fetal growth and one of the most prominent and significantly disordered features of FGR. With correction of brain sparing and increased growth in some of these peripheral organs with our *IGF1* therapy we may be able to reduce the probability of developing many cognitive and peripheral organ morbidities later in life.

Appropriate fetal development and growth is dependent on the transfer of oxygen and nutrients from the maternal to fetal circulation. Blood glucose and oxygen are reduced in FGR fetuses often as a result of aberrant placenta structure and/or function, impeding oxygen diffusion or failing to transport enough nutrients into fetal circulation [54-56]. Glucose is the main source of energy for almost every tissue type in the body [57]. Fetal metabolism and growth will impact an individual's body functions and energy metabolism for the rest of their lives, so if a fetus is receiving less than adequate levels of energy then all the organ systems of the body are put under stress. In our model, MNR male fetal blood glucose is significantly reduced compared to controls. Our treatment, however, corrects these decreased levels with both direct and indirect *IGF1* treatment to the male placentas. Blood glucose was not statistically changed with MNR or nanoparticle-mediated *IGF1* treatment in females, possibly due to the females having less changes in endogenous *gpIgf1* levels as well as their compensatory *Igf2* signaling changes with

MNR and therefore not leading to significant changes in glucose transport. Lactate is the end product of anaerobic metabolism of glucose and many studies have shown that it is produced in the placenta and delivered to both the maternal and fetal circulations. This lactate is used for oxidation, and increased levels of lactate have been correlated with fetal oxygenation and FGR [58]. FGR fetuses have higher lactate levels than AGA fetuses as seen in our female MNR fetuses [59]. Indirect exposure of the female placentas was not able to reduce these elevated fetal blood lactate levels, however the impact of direct *IGF1* treatment in a female placenta may differ. In this study we demonstrate changes in blood glucose and lactate levels with placental insufficiency, and correction of blood glucose levels with our nanoparticle-mediated *IGF1* treatment.

It is well established that increased cortisol in pregnancy can cause disruption to fetal organ development and metabolism that leads to increased disease risk later in life, a process described as fetal programming (to which the entire DOHaD field is dedicated to understanding these mechanisms) [60-64]. In MNR fetuses of both sexes we identified statistically significant increases in cortisol levels. This elevation is likely due to a combination of the fetus receiving less than adequate levels of nutrients for proper growth and reduced handling of maternal cortisol in the insufficient FGR placentas as seen in human cases that leads to increased cortisol crossing the placenta and impacting fetal organ development [64-67]. Several studies have found increased maternal and fetal cortisol levels during pregnancy lead to increased risk of cognitive delays in the offspring, metabolic diseases, and cardiovascular disorders [60, 68, 69]. Here, we induce biologically relevant reductions of cortisol in both fetuses whose placentas were indirectly exposed, and complete reduction to control levels in fetuses whose placentas received direct nanoparticle-mediated *IGF1* treatment. This correction of fetal cortisol shows immense potential for creating a better pregnancy environment that could lead to reduced risks for countless multigenerational adult morbidities creating healthier humans and vastly decreasing medical care needs and expenses.

It is equally important to ascertain the impact of this treatment on maternal physiology. Maternal blood measures included in this study; glucose, lactate, potassium, sodium and progesterone, represent commonly and easily measurable biochemical assays throughout human pregnancy. Our aim was to understand the impact of our treatment, if any, on the maternal system as aberrant changes in these levels could indicate negative impact on maternal physiology. We showed that glucose, lactate, sodium, potassium, and progesterone levels remained unchanged across all groups. Unsurprisingly, as our MNR model and human FGR have been linked to increased maternal stress, the maternal cortisol levels were elevated. In humans, elevated maternal cortisol not only may cross the insufficient placenta but also cause vasoconstriction of the uterine arteries leading to reduced blood flow in the placenta and poor fetal growth [70]. Furthermore, maternal cortisol increase with FGR in humans has been linked to higher risks of developing postpartum depression, coronary heart disease, and post-traumatic stress disorder [64, 71]. This is the first study to demonstrate that with treatment of the placenta, however, full correction of maternal cortisol levels back to control levels. While more understanding of the underlying mechanism is needed to fully understand these changes, the dams are still on a restricted diet which indicates reduced maternal cortisol is likely due to improved placental handling of the cortisol and restored placental signaling to the maternal system. This is the first report showing a

placental therapy for FGR with the capability of improving the fetal, placental, and maternal environments.

A limitation of this study was the lack of female fetuses receiving direct injection of our nanoparticle-mediated *IGF1*. We have previously shown that all placentas in the litter express the plasmid-specific *hIGF1* following a single nanoparticle-mediated *IGF1* injection into one placenta [23], and chose to continue this approach. However, fetal sex cannot reliably be determined via ultrasound at mid-pregnancy and prior to our gene therapy delivery, hence we cannot control for which sex gets directly treated. Studies in non-human primates which carry a singleton pregnancy are underway to assess translational aspects including dose, delivery and safety and will address both fetal sexes. Further mechanistic studies in the guinea pig into precise sexual dimorphic responses to the nanoparticle-mediated *IGF1* will also be conducted as sex as a biological variable is a pivotal factor in all aspects of fetal and placental development.

In the present study, for the first time, we show the ability of our novel nanoparticle-mediated *IGF1* gene therapy to restore fetal growth to levels comparable with control with repeated treatment in a guinea pig model. Positive changes to fetal growth trajectories were associated with increased fetal blood glucose and reduced fetal cortisol, indicative of improved fetal physiology. Additionally, we showed no change in any maternal physiological measures while also correcting increased blood cortisol levels. We show here for the first time a therapy capable of improving the entire pregnancy environment: maternal, placental, and fetal. By improving each of these environments we may be able to help improve the health of mothers and offspring through the rest of their lives. This combined with our previous studies using this therapy at both mid pregnancy and in numerous cell and animal models demonstrate the plausibility of this therapy for future human translation. By correcting fetal growth restriction and asymmetric growth we have the potential to prevent countless stillbirths, reduce preterm birth rates, decrease long NICU stays, and decrease numerous morbidities associated with the developmental origins of disease for overall healthier lives.

Data availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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